MUTATIONS IN ION CHANNELS

Technical Field

The present invention is concerned with mutations in proteins having biological functions as ion channels and, more particularly, with such mutations where they are associated with diseases such as epilepsy and disorders associated with ion channel dysfunction including, but not restricted to, hyper- or hypo-kalemic periodic paralysis, myotonias, malignant hyperthermia, myasthenia, cardiac arrhythmias, episodic ataxia, migraine, Alzheimer's disease, Parkinson's disease, schizophrenia, hyperekplexia, anxiety, depression, phobic obsessive symptoms, neuropathic pain, inflammatory pain, chronic/acute pain, Bartter's syndrome, polycystic kidney disease, Dent's disease, hyperinsulinemic hypoglycemia of infancy, cystic fibrosis, congenital stationary night blindness and total colour-blindness.

Background Art

Epilepsies constitute a diverse collection of brain disorders that affect about 3% of the population at some time in their lives (Annegers, 1996). An epileptic seizure can be defined as an episodic change in behaviour caused by the disordered firing of populations of neurons in the central nervous system. This results in varying degrees of involuntary muscle contraction and often a loss of consciousness. Epilepsy syndromes have been classified into more than 40 distinct types based upon characteristic symptoms, types of seizure, cause, age of onset and EEG patterns (Commission on Classification and Terminology of the International League Against Epilepsy, 1989). However the single feature that is common to all syndromes is the persistent increase in neuronal excitability that is both occasionally and unpredictably expressed as a seizure.

A genetic contribution to the aetiology of epilepsy has been estimated to be present in approximately 40% of

affected individuals (Gardiner, 2000). As epileptic seizures may be the end-point of a number of molecular aberrations that ultimately disturb neuronal synchrony, the genetic basis for epilepsy is likely to be heterogeneous. There are over 200 Mendelian diseases which include epilepsy as part of the phenotype. In these diseases, seizures are symptomatic of underlying neurological involvement such as disturbances in brain structure or function. In contrast, there are also a number of "pure" epilepsy syndromes in which epilepsy is the sole manifestation in the affected individuals. These are termed idiopathic and account for over 60% of all epilepsy cases.

Idiopathic epilepsies have been further divided into partial and generalized sub-types. Partial (focal or local) epileptic fits arise from localized cortical discharges, so that only certain groups of muscles are involved and consciousness may be retained. However, in generalized epilepsy, EEG discharge shows no focus such that all subcortical regions of the brain are involved. Although the observation that generalized epilepsies are frequently inherited is understandable, the mechanism by which genetic defects, presumably expressed constitutively in the brain, give rise to partial seizures is less clear.

The molecular genetic era has resulted in spectacular advances in classification, diagnosis and biological understanding of numerous inherited neurological disorders including muscular dystrophies, familial neuropathies and spinocerebellar degenerations. These disorders are all uncommon or rare and have simple Mendelian inheritance. In contrast, common neurological diseases like epilepsy, have complex inheritance where they are determined by multiple genes sometimes interacting with environmental influences. Molecular genetic advances in disorders with complex inheritance have been far more modest to date (Todd, 1999).

Most of the molecular genetic advances have occurred by a sequential three stage process. First a clinically homogeneous disorder is identified and its mode of inheritance determined. Second, linkage analysis is performed on carefully characterized clinical populations with the disorder. Linkage analysis is a process where the chromosomal localization of a particular disorder is narrowed down to approximately 0.5% of the total genome. Knowledge of linkage imparts no intrinsic biological insights other than the important clue as to where to look in the genome for the abnormal gene. Third, strategies such as positional cloning or the positional candidate approach are used to identify the aberrant gene and its specific mutations within the linked region (Collins, 1995).

Linkage studies in disorders with complex inheritance have been bedevilled by negative results and by failure to replicate positive findings. A sense of frustration permeates current literature in the genetics of complex disorders. Carefully performed, large scale studies involving hundreds of sibpairs in disorders including multiple sclerosis and diabetes have been essentially negative (Bell and Lathrop, 1996; Lernmark and Ott, 1998). An emerging view is that such disorders are due to the summation of many genes of small effect and that identification of these genes may only be possible with very large-scale association studies. Such studies on a genome-wide basis are currently impossible due to incomplete marker sets and computational limitations.

The idiopathic generalized epilepsies (IGE) are the most common group of inherited human epilepsy and do not have simple inheritance. Like other complex disorders, linkage studies in IGE have generated controversial and conflicting claims. Previous authors have suggested the possibility of multifactorial, polygenic, oligogenic or two locus models for the disease (Andermann, 1982; Doose

and Baier, 1989; Greenberg et al., 1988*a*; 1992; Janz et al., 1992).

Two broad groups of IGE are now known – the classical idiopathic generalized epilepsies (Commission on Classification and Terminology of the International League Against Epilepsy, 1989) and the newly recognized genetic syndrome of generalized epilepsy with febrile seizures plus ($GEFS^+$) (Scheffer and Berkovic, 1997; Singh et al., 1999).

The classical IGEs are divided into a number of clinically recognizable but overlapping sub-syndromes including childhood absence epilepsy, juvenile absence epilepsy, juvenile myoclonic epilepsy etc (Commission on Classification and Terminology of the International League Against Epilepsy, 1989; Roger et al., 1992). The sub-syndromes are identified by age of onset and the pattern of seizure types (absence, myoclonus and tonic-clonic). Some patients, particularly those with tonic-clonic seizures alone do not fit a specifically recognized sub-syndrome. Arguments for regarding these as separate syndromes, yet recognizing that they are part of a neurobiological continuum, have been presented previously (Berkovic et al. 1987; 1994; Reutens and Berkovic, 1995).

$GEFS^+$ was originally recognized through large multi-generation families and comprises a variety of sub-syndromes. Febrile seizures plus (FS^+) is a sub-syndrome where children have febrile seizures occurring outside the age range of 3 months to 6 years, or have associated febrile tonic-clonic seizures. Many family members have a phenotype indistinguishable from the classical febrile convulsion syndrome and some have FS^+ with additional absence, myoclonic, atonic, or complex partial seizures. The severe end of the $GEFS^+$ spectrum includes myoclonic-astatic epilepsy.

The cumulative incidence for epilepsy by age 30 years (proportion suffering from epilepsy at some time) is 1.5% (Hauser et al., 1993). Accurate estimates for the

cumulative incidence of the IGEs are unavailable. In epidemiological studies where attempts are made to subclassify epilepsies, rather few cases of IGE are diagnosed, and many cases are unclassified. This is probably because cases are rarely directly examined by epileptologists. In clinic- or office-based series seen by experts, most cases are classifiable and IGEs account for about 25% of cases. This suggests that about 0.3% of the population suffer from IGE at some time.

In outbred populations many patients with classical IGE appear to be sporadic as siblings and parents are usually unaffected. Systematic EEG studies on clinically unaffected family members show an increase in age-dependent occurrence of generalized epileptiform discharges compared to controls. In addition, to the approximate 0.3% of the population with clinical IGE, systematic EEG studies suggest that about 1% of healthy children have generalized epileptiform discharges while awake (Cavazzuti et al., 1980; Okubo et al., 1994).

Approximately 5-10% of first degree relatives of classical IGE probands have seizures with affected relatives usually having IGE phenotypes or febrile seizures. While nuclear families with 2-4 affected individuals are well recognized and 3 generation families or grandparent-grandchild pairs are occasionally observed (Italian League Against Epilepsy Genetic Collaborative Group, 1993), families with multiple affected individuals extending over 4 or more generations are exceptionally rare.

For GEFS$^+$, however, a number of large multi-generation families showing autosomal dominant inheritance with incomplete penetrance are known. Similar to classical IGE, analysis of sporadic cases and small families with GEFS$^+$ phenotypes does not suggest simple Mendelian inheritance. Indeed, bilineal inheritance, where there is a history of epilepsy on maternal and paternal sides, is observed in both GEFS$^+$ and classical IGE families (Singh et al., 1999;

Italian League Against Epilepsy Genetic Collaborative Group, 1993).

Within single families with classical IGE or GEFS$^+$, affected individuals often have different sub-syndromes. The closer an affected relative is to the proband, the more similar are their sub-syndromes, and siblings often have similar sub-syndromes (Italian League Against Epilepsy Genetic Collaborative Group, 1993). Less commonly, families are observed where most, or all, known affected individuals have one classical IGE sub-syndrome such as childhood absence epilepsy or juvenile myoclonic epilepsy (Italian League Against Epilepsy Genetic Collaborative Group, 1993).

Importantly, sub-syndromes are identical in affected monozygous twins with IGE. In contrast, affected dizygous twins, may have the same or different sub-syndromes. Classical IGE and GEFS$^+$ sub-syndromes tend to segregate separately (Singh et al., 1999).

In some inbred communities, pedigree analysis strongly suggests recessive inheritance for juvenile myoclonic epilepsy and other forms of IGE (Panayiotopoulos and Obeid, 1989; Berkovic et al., 2000). In such families, sub-syndromes are much more similar in affected siblings than in affected sib-pairs from outbred families. Recently, a family with an infantile form of IGE with autosomal recessive inheritance, confirmed by linkage analysis, was described in Italy (Zara et al., 2000).

Most work on the molecular genetics of classical IGEs has been done on the sub-syndrome of juvenile myoclonic epilepsy where a locus in proximity or within the HLA region on chromosome 6p was first reported in 1988 (Greenberg et al., 1988*b*). This finding was supported by two collaborating laboratories, in separate patient samples, and subsequently three groups provided further evidence for a 6p locus for juvenile myoclonic epilepsy in some, but not all, of their families. However, genetic defects have not been found and the exact locus of the

gene or genes, in relationship to the HLA region, remains controversial. Strong evidence for linkage to chromosome 6 also comes from a study of a single large family with juvenile myoclonic epilepsy, but in this pedigree the locus is well outside the HLA region. A locus on chromosome 15q has also been suggested for juvenile myoclonic epilepsy, but was not confirmed by two other studies.

In general, the results of studies of the putative chromosomal 6p locus in the HLA region in patients with absence epilepsies or other forms of idiopathic generalized epilepsies have been negative. The major exception is that study of probands with tonic-clonic seizures on awakening, a sub-syndrome closely related to juvenile myoclonic epilepsy, suggests linkage to 6p.

Linkage for classical remitting childhood absence epilepsy remains elusive, but in a family with persisting absence evolving into a juvenile myoclonic epilepsy phenotype, linkage to chromosome 1p has been claimed. An Indian pedigree with persisting absence and tonic-clonic seizures may link to 8q24. Linkage to this region was also suggested by a non-parametric analysis in IGE, irrespective of subsyndrome, but was not confirmed in another study. Other loci for IGEs that have been reported in single studies include 3p14, 8p, 18 and possibly 5p. The unusual example of recessively inherited infantile onset IGE described in Italy maps to 16p in a single family.

Thus, like most disorders with complex inheritance, the literature on genetics of classical IGEs is confusing and contradictory. Some, and perhaps much, of this confusion is due to heterogeneity, with the likelihood of a number of loci for IGEs. The studies reviewed above were principally performed on multiple small families, so heterogeneity within and between samples is probable. Whether all, some, or none of the linkages reported above will be found to harbour relevant genes for IGE remains to

be determined. Most of the studies reviewed above used analysis methods assuming Mendelian inheritance, an assumption that is not correct for outbred communities. Some studies used multiple models (autosomal recessive, autosomal dominant). Although parametric linkage analysis may be reliable in some circumstance of analyzing complex disease, it can lead to spurious findings as highlighted by the literature on linkage in major psychoses (Risch and Botstein, 1996).

In so far as $GEFS^+$ is concerned, linkage analysis on rare multi-generation large families with clinical evidence of a major autosomal dominant gene have demonstrated loci on chromosomes 19q and 2q. Both the 19q and 2q $GEFS^+$ loci have been confirmed in independently ascertained large families, and genetic defects have been identified. Families linked to 19q are known and a mutation in the gene for the β1 subunit of the neuronal sodium channel (SCN1B) has been identified (Wallace et al., 1998). This mutation results in the loss of a critical disulphide bridge of this regulatory subunit and causes a loss of function *in vitro*. Families linked to 2q are also known and mutations in the pore-forming α subunit of the neuronal sodium channel (SCN1A) have been identified (PCT/AU01/01648; Wallace et al., 2001*b*; Escayg et al., 2000). Studies on the more common small families with $GEFS^+$ have not revealed these or other mutations to date.

In addition to the *SCN1B* and *SCN1A* mutations in $GEFS^+$, four other gene defects have been discovered for human idiopathic epilepsies through the study of large families. Mutations in the alpha-4 subunit of the neuronal nicotinic acetylcholine receptor (CHRNA4) occur in the focal epilepsy syndrome of autosomal dominant nocturnal frontal lobe epilepsy (Australian patent AU-B-56247/96; Steinlein et al., 1995). Mutations in the gamma-2 subunit of the $GABA_A$ receptor (GABRG2) have been identified in childhood absence epilepsy, febrile seizures (including febrile

seizures plus) and myoclonic epilepsy (PCT/AU01/00729; Wallace et al., 2001a). Finally, mutations in two potassium channel genes (KCNQ2 and KCNQ3) were identified in benign familial neonatal convulsions (Singh et al., 1998; Biervert et al., 1998; Charlier et al., 1998). Although initially regarded as a special form of IGE, this unusual syndrome is probably a form of inherited focal epilepsy.

Further to these studies, mutations in other genes have been identified to be causative of epilepsy. These include mutations in the beta-2 subunit (CHRNB2) of the neuronal nicotinic acetylcholine receptor (PCT/AU01/00541; Phillips et al., 2001) and the delta subunit (GABRD) of the $GABA_A$ receptor (PCT/AU01/00729).

A number of mouse models approximating human IGE are known. These mice mutants have ataxia in addition to generalized spike-and-wave discharges with absences or tonic-clonic seizures. Recessive mutations in calcium channel subunit genes have been found in lethargic (CACNB4), tottering/leaner (CACNA1A), and stargazer (CACNG2) mutants. The slow-wave epilepsy mouse mutant has a mutation in the sodium/hydrogen exchanger gene, which may have important downstream effects on pH-sensitive ion channels.

The human and mouse literature is now suggesting that the idiopathic epilepsies comprise a family of channelopathies with mutations in ion channel subunits of voltage-gated (eg SCN1A, SCN1B, KCNQ2, KCNQ3) or ligand-gated (eg CHRNA4, CHRNB2, GABRG2, GABRD) types. These channels are typically comprised of a number of subunits, specified by genes on different chromosomes. The stoichiometry and conformation of ion channel subunits are not yet well understood, but many have multiple subunits in a variety of combinations.

The involvement of ion channels in other neuro/physiological disorders has also been observed (reviewed in Dworakowska and Dolowy, 2000). Mutations in

voltage-gated sodium, potassium, calcium and chloride channels as well as ligand-gated channels such as the acetylcholine and GABA receptors may lead to physiological disorders such as hyper- and hypo-kalemic periodic paralysis, myotonias, malignant hyperthermia, myasthenia and cardiac arrhythmias. Neurological disorders other than epilepsy that are associated with ion channel mutations include episodic ataxia, migraine, Alzheimer's disease, Parkinson's disease, schizophrenia, hyperekplexia, anxiety, depression, phobic obsessive symptoms, as well as neuropathic pain, inflammatory pain and chronic/acute pain. Some kidney disorders such as Bartter's syndrome, polycystic kidney disease and Dent's disease, secretion disorders such as hyperinsulinemic hypoglycemia of infancy and cystic fibrosis, and vision disorders such as congenital stationary night blindness and total colour-blindness may also be linked to mutations in ion channels.

Disclosure of the Invention

In a new genetic model for the idiopathic generalised epilepsies (IGEs) described in PCT/AU01/00872 (the disclosure of which is incorporated herein by reference) it has been postulated that most classical IGE and GEFS$^+$ cases are due to the combination of two mutations in multi-subunit ion channels. These are typically point mutations resulting in a subtle change of function. The critical postulate is that two mutations, usually, but not exclusively, in different subunit alleles ("digenic model"), are required for clinical expression of IGE. It was further proposed that

a) A number of different mutated subunit pairs can be responsible for IGE. Combinations of two mutated subunits lead to an IGE genotype with ~30% penetrance.

b) The total allele frequency of mutated subunits is ~8%. It was calculated that approximately 15% of the population has one or more mutated

subunit genes and 1% have two or more mutated subunits.

c) Sub-syndromes are principally determined by the specific combination of mutated subunit pairs, although one or more other genes, including ion channel subunits, of smaller effect may modify the phenotype.

d) Mutated subunit combinations that cause classical IGEs are largely separate from those that cause GEFS$^+$, although some subunits may be involved in both syndromes.

e) Individuals with single 'change of function' mutations would not have IGE, but such mutations may contribute to simple febrile seizures, which are observed with increased frequency in relatives of IGE probands.

The model also proposes that subunit mutations with more severe functional consequences (eg breaking a disulphide bridge in SCN1B or amino acid substitution in the pore forming regions of SCN1A for GEFS$^+$) cause autosomal dominant generalized epilepsies with a penetrance of 60-90%. The precise sub-syndromes in GEFS$^+$ are determined by minor allelic variation or mutations in other ion channel subunits. Such "severe" mutations are rare (allele frequency <0.01%) and are infrequent causes of GEFS$^+$. They very rarely, or perhaps never, cause classical IGE.

The identification of molecular changes in ion channel subunits is therefore a significant step towards the elucidation of genetic variants that alone or in combination (based on the digenic model) give rise to an epilepsy phenotype, and to other neuro/physiological disorders associated with ion channel dysfunction.

The present inventors have identified a number of novel mutations or variants in genes encoding subunits of ion channels in individuals with epilepsy. It will be appreciated that for each molecular defect one can provide

an isolated nucleic acid molecule coding for a protein having a biological function as part of an ion channel in a mammal, wherein a mutation event selected from the group consisting of point mutations, deletions, insertions and rearrangements has occurred so as to affect the functioning of the ion channel. In some instances this single mutation alone will produce a phenotype of epilepsy or other neuro/physiological disorders associated with ion channel dysfunction.

In the case where a single mutation alone does not produce, say, an epilepsy phenotype, there would be provided one or more additional isolated nucleic acid molecules coding for proteins having a biological function as part of an ion channel in a mammal, wherein a mutation event selected from the group consisting of point mutations, deletions, insertions and rearrangements has occurred so as to affect the functioning of the ion channel. The cumulative effect of the mutations in each isolated nucleic acid molecule *in vivo* is to produce a epilepsy or another neuro/physiological disorders in said mammal. The mutations may be in nucleic acid molecules coding for protein subunits belonging to the same ion channel or may be in nucleic acid molecules coding for protein subunits that belong to different ion channels.

Typically such mutations are point mutations and the ion channels are voltage-gated channels such as a sodium, potassium, calcium or chloride channels or are ligand-gated channels such as members of the nAChR/GABA super family of receptors, or a functional fragment or homologue thereof.

Mutations may include those in non-coding regions of the ion channel subunits (eg mutations in the promoter region which affect the level of expression of the subunit gene, mutations in intronic sequences which affect the correct splicing of the subunit during mRNA processing, or mutations in the 5′ or 3′ untranslated regions that can affect translation or stability of the mRNA). Mutations

may also and more preferably will be in coding regions of the ion channel subunits (eg nucleotide mutations may give rise to an amino acid change in the encoded protein or nucleotide mutations that do not give rise to an amino acid change but may affect the stability of the mRNA).

Mutation combinations may be selected from, but are not restricted to, those identified in Table 1.

Accordingly in one aspect of the present invention there is provided a method of identifying a subject predisposed to a disorder associated with ion channel dysfunction, comprising ascertaining whether at least one of the genes encoding ion channel subunits in said subject has undergone a mutation event selected from the group consisting of the mutation events set forth in the following Table:

Subunit Gene	Exon/Intron	DNA Mutation
SCN1A	Exon 5	c664C→T
SCN1A	Exon 8	c1152G→A
SCN1A	Exon 9	c1183G→C
SCN1A	Exon 9	c1207T→C
SCN1A	Exon 9	c1237T→A
SCN1A	Exon 9	c1265T→A
SCN1A	Exon 21	c4219C→T
SCN1A	Exon 26	c5339T→C
SCN1A	Exon 26	c5674C→T
SCN1B	Exon 3	c254G→A
SCN2A	Exon 6A	c668G→A
SCN2A	Exon 16	c2674G→A
SCN2A	Exon 17	c3007C→A
SCN2A	Exon 19	c3598A→G
SCN2A	Exon 20	c3956G→A
SCN2A	Exon 12	c1785T→C
SCN2A	Exon 27	c4919T→A
SCN1A	Intron 9	IVS9-1G→A
SCN1A	Intron 23	IVS23+33G→A
SCN2A	Intron 7	IVS7+61T→A
SCN2A	Intron 19	IVS19-55A→G
SCN2A	Intron 22	IVS22-31A→G
SCN2A	Intron 2	IVS2-28G→A
SCN2A	Intron 8	IVS8-3T→C
SCN2A	Intron 11	IVS11+49A→G
SCN2A	Intron 11	IVS11-16C→T

SCN2A	Intron 17	IVS17-71C→T
SCN2A	Intron 17	IVS17-74delG
SCN2A	Intron 17	IVS17-74insG
CHRNA5	Exon 4	c400G→A
CHRNA2	Exon 4	c373G→A
CHRNA3	Exon 2	c110G→A
CHRNA2	Exon 4	c351C→T
CHRNA2	Exon 5	c771C→T
CHRNA3	Exon 2	c159A→G
CHRNA3	Exon 4	c291G→A
CHRNA3	Exon 4	c345G→A
CHRNA2	Intron 3	IVS3-16C→T
CHRNA3	Intron 3	IVS3-5T→C
CHRNA3	Intron 4	IVS4+8G→C
KCNQ2	Exon 1	c204-c205insC
KCNQ2	Exon 1	c1A→G
KCNQ2	Exon 1	c2T→C
KCNQ2	Exon 8	c1057C→G
KCNQ2	Exon 11	c1288C→T
KCNQ2	Exon 14	c1710A→T
KCNQ2	Exon 15	c1856T→G
KCNQ2	Intron 9	IVS9+(46-48)delCCT
KCNQ3	Intron 11	IVS11+43G→A
KCNQ3	Intron 12	IVS12+29G→A
GABRB1	Exon 5	c508C→T
GABRB1	Exon 9	c1329G→A
GABRB1	Exon 8	c975C→T
GABRG3	Exon 8	c995T→C
GABRA1	5′ UTR	c-142A→G
GABRA1	5′ UTR	c-31C→T
GABRA2	3′ UTR	c1615G→A
GABRA5	5′ UTR	c-271G→C
GABRA5	5′ UTR	c-228A→G
GABRA5	5′ UTR	c-149G→C
GABRB2	5′ UTR	c-159C→T
GABRB2	3′ UTR	c1749C→T
GABRPi	5′ UTR	c-101C→T
GABRB1	Intron 1	IVS1+24T→G
GABRB1	Intron 6	IVS6+72T→G
GABRB1	Intron 7	IVS7-34A→G
GABRB3	Intron 1	IVS1-14C→T
GABRB3	Intron 7	IVS7+58delAA
GABRD	Intron 6	IVS6+132insC
GABRD	Intron 6	IVS6+130insC
GABRD	Intron 6	IVS6+73delCGCGCCCACCGCCCCTTCCGCG
GABRG3	Intron 8	IVS8-102C→T

In a further aspect there is provided a method of identifying a subject predisposed to a disorder associated with ion channel dysfunction, comprising ascertaining whether at least one of the genes encoding ion channel subunits in said subject has undergone a mutation event as set forth in one of SEQ ID Numbers: 1-72.

In another aspect of the present invention there is provided an isolated nucleic acid molecule encoding a mutant or variant ion channel subunit wherein a mutation event selected from the group consisting of the mutation events set forth in the following Table:

Subunit Gene	Exon/Intron	DNA Mutation
SCN1A	Exon 5	c664C→T
SCN1A	Exon 8	c1152G→A
SCN1A	Exon 9	c1183G→C
SCN1A	Exon 9	c1207T→C
SCN1A	Exon 9	c1237T→A
SCN1A	Exon 9	c1265T→A
SCN1A	Exon 21	c4219C→T
SCN1A	Exon 26	c5339T→C
SCN1A	Exon 26	c5674C→T
SCN1B	Exon 3	c254G→A
SCN2A	Exon 6A	c668G→A
SCN2A	Exon 16	c2674G→A
SCN2A	Exon 17	c3007C→A
SCN2A	Exon 19	c3598A→G
SCN2A	Exon 20	c3956G→A
SCN2A	Exon 12	c1785T→C
SCN2A	Exon 27	c4919T→A
SCN1A	Intron 9	IVS9-1G→A
SCN1A	Intron 23	IVS23+33G→A
SCN2A	Intron 7	IVS7+61T→A
SCN2A	Intron 19	IVS19-55A→G
SCN2A	Intron 22	IVS22-31A→G
SCN2A	Intron 2	IVS2-28G→A
SCN2A	Intron 8	IVS8-3T→C
SCN2A	Intron 11	IVS11+49A→G
SCN2A	Intron 11	IVS11-16C→T
SCN2A	Intron 17	IVS17-71C→T
SCN2A	Intron 17	IVS17-74delG
SCN2A	Intron 17	IVS17-74insG
CHRNA5	Exon 4	c400G→A
CHRNA2	Exon 4	c373G→A

CHRNA3	Exon 2	c110G→A
CHRNA2	Exon 4	c351C→T
CHRNA2	Exon 5	c771C→T
CHRNA3	Exon 2	c159A→G
CHRNA3	Exon 4	c291G→A
CHRNA3	Exon 4	c345G→A
CHRNA2	Intron 3	IVS3-16C→T
CHRNA3	Intron 3	IVS3-5T→C
CHRNA3	Intron 4	IVS4+8G→C
KCNQ2	Exon 1	c204-c205insC
KCNQ2	Exon 1	c1A→G
KCNQ2	Exon 1	c2T→C
KCNQ2	Exon 8	c1057C→G
KCNQ2	Exon 11	c1288C→T
KCNQ2	Exon 14	c1710A→T
KCNQ2	Exon 15	c1856T→G
KCNQ2	Intron 9	IVS9+(46-48)delCCT
KCNQ3	Intron 11	IVS11+43G→A
KCNQ3	Intron 12	IVS12+29G→A
GABRB1	Exon 5	c508C→T
GABRB1	Exon 9	c1329G→A
GABRB1	Exon 8	c975C→T
GABRG3	Exon 8	c995T→C
GABRA1	5′ UTR	c-142A→G
GABRA1	5′ UTR	c-31C→T
GABRA2	3′ UTR	c1615G→A
GABRA5	5′ UTR	c-271G→C
GABRA5	5′ UTR	c-228A→G
GABRA5	5′ UTR	c-149G→C
GABRB2	5′ UTR	c-159C→T
GABRB2	3′ UTR	c1749C→T
GABRPi	5′ UTR	c-101C→T
GABRB1	Intron 1	IVS1+24T→G
GABRB1	Intron 6	IVS6+72T→G
GABRB1	Intron 7	IVS7-34A→G
GABRB3	Intron 1	IVS1-14C→T
GABRB3	Intron 7	IVS7+58delAA
GABRD	Intron 6	IVS6+132insC
GABRD	Intron 6	IVS6+130insC
GABRD	Intron 6	IVS6+73delCGCGCCCACCGCCCCTTCCGCG
GABRG3	Intron 8	IVS8-102C→T

has occurred.

In still another aspect of the present invention there is provided an isolated nucleic acid molecule encoding a mutant or variant ion channel subunit wherein a

mutation event has occurred as set forth in one of SEQ ID Numbers: 1-72.

The mutation event disrupts the functioning of an ion channel so as to produce a phenotype of epilepsy, and/or one or more other disorders associated with ion channel dysfunction, including but not restricted to, hyper- or hypo-kalemic periodic paralysis, myotonias, malignant hyperthermia, myasthenia, cardiac arrhythmias, episodic ataxia, migraine, Alzheimer's disease, Parkinson's disease, schizophrenia, hyperekplexia, anxiety, depression, phobic obsessive symptoms, neuropathic pain, inflammatory pain, chronic/acute pain, Bartter's syndrome, polycystic kidney disease, Dent's disease, hyperinsulinemic hypoglycemia of infancy, cystic fibrosis, congenital stationary night blindness and total colour-blindness, either alone or in combination with one or more additional mutations or variations in the ion channel subunit genes.

In another aspect of the present invention there is provided an isolated nucleic acid molecule encoding a mutant KCNQ2 subunit, wherein the mutation event has occurred in the C-terminal domain of the KCNQ2 subunit and leads to a disturbance in the calmodulin binding affinity of the subunit, so as to produce an epilepsy phenotype.

In one form of the invention, the mutations are in exon 8 or exon 15 of the KCNQ2 subunit and result in the replacement of an arginine residue with a glycine residue at amino acid position 353, or the replacement of a leucine residue with an arginine at amino acid position 619. The R353G mutation occurs as a result of a C to G nucleotide substitution at position 1057 of the KCNQ2 coding sequence as shown in SEQ ID NO: 44. The L619R mutation occurs as a result of a T to G nucleotide substitution at position 1856 of the KCNQ2 coding sequence as shown in SEQ ID NO: 47.

In a further form of the invention, the mutations are in exon 11 or exon 14 of the KCNQ2 subunit and result in

the replacement of an arginine residue with a stop codon at amino acid position 430, or the replacement of an arginine residue with a serine at amino acid position 570. The R430X mutation occurs as a result of a C to T nucleotide substitution at position 1288 of the KCNQ2 coding sequence as shown in SEQ ID NO: 45. The R570S mutation occurs as a result of an A to T nucleotide substitution at position 1710 of the KCNQ2 coding sequence as shown in SEQ ID NO: 46.

Preferably these mutations create a phenotype of benign familial neonatal seizures (BFNS).

In a further aspect of the present invention there is provided a combination of two or more isolated nucleic acid molecules each having a novel mutation event as laid out in Table 1. The cumulative effect of the mutations in each isolated nucleic acid molecule *in vivo* is to produce an epilepsy or another disorder associated with ion channel dysfunction as described above in said mammal.

In a particularly preferred embodiment of the present invention, the isolated nucleic acid molecules have a nucleotide sequence as shown in any one of SEQ ID Numbers: 1-72. The sequences correspond to the novel DNA mutations or variants laid out in Table 1.

In another aspect of the present invention there is provided an isolated nucleic acid molecule comprising any one of the nucleotide sequences set forth in SEQ ID Numbers: 1-72.

In another aspect of the present invention there is provided an isolated nucleic acid molecule consisting of any one of the nucleotide sequences set forth in SEQ ID Numbers: 1-72.

The nucleotide sequences of the present invention can be engineered using methods accepted in the art for a variety of purposes. These include, but are not limited to, modification of the cloning, processing, and/or expression of the gene product. PCR reassembly of gene fragments and the use of synthetic oligonucleotides allow

the engineering of the nucleotide sequences of the present invention. For example, oligonucleotide-mediated site-directed mutagenesis can introduce further mutations that create new restriction sites, alter expression patterns and produce splice variants etc.

As a result of the degeneracy of the genetic code, a number of polynucleotide sequences, some that may have minimal similarity to the polynucleotide sequences of any known and naturally occurring gene, may be produced. Thus, the invention includes each and every possible variation of a polynucleotide sequence that could be made by selecting combinations based on possible codon choices. These combinations are made in accordance with the standard triplet genetic code as applied to the polynucleotide sequences of the present invention, and all such variations are to be considered as being specifically disclosed.

The nucleic acid molecules of this invention are typically DNA molecules, and include cDNA, genomic DNA, synthetic forms, and mixed polymers, both sense and antisense strands, and may be chemically or biochemically modified, or may contain non-natural or derivatised nucleotide bases as will be appreciated by those skilled in the art. Such modifications include labels, methylation, intercalators, alkylators and modified linkages. In some instances it may be advantageous to produce nucleotide sequences possessing a substantially different codon usage than that of the polynucleotide sequences of the present invention. For example, codons may be selected to increase the rate of expression of the peptide in a particular prokaryotic or eukaryotic host corresponding with the frequency that particular codons are utilized by the host. Other reasons to alter the nucleotide sequence without altering the encoded amino acid sequences include the production of RNA transcripts having more desirable properties, such as a greater half-

life, than transcripts produced from the naturally occurring mutated sequence.

The invention also encompasses production of nucleic acid sequences of the present invention entirely by synthetic chemistry. Synthetic sequences may be inserted into expression vectors and cell systems that contain the necessary elements for transcriptional and translational control of the inserted coding sequence in a suitable host. These elements may include regulatory sequences, promoters, 5' and 3' untranslated regions and specific initiation signals (such as an ATG initiation codon and Kozak consensus sequence) which allow more efficient translation of sequences encoding the polypeptides of the present invention. In cases where the complete coding sequence, including the initiation codon and upstream regulatory sequences, are inserted into the appropriate expression vector, additional control signals may not be needed. However, in cases where only coding sequence, or a fragment thereof, is inserted, exogenous translational control signals as described above should be provided by the vector. Such signals may be of various origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of enhancers appropriate for the particular host cell system used (Scharf et al., 1994).

The invention also includes nucleic acid molecules that are the complements of the sequences described herein.

The present invention allows for the preparation of purified polypeptide or protein from the polynucleotides of the present invention, or variants thereof. In order to do this, host cells may be transformed with a novel nucleic acid molecule as described above, or with nucleic acid molecules encoding two or more mutant ion channel subunits. If the mutant subunits form a part of the same ion channel a receptor protein containing two or more mutant subunits may be isolated. If the mutant subunits are subunits of different ion channels the host cells will

express two or more mutant receptor proteins. Typically said host cells are transfected with an expression vector comprising a DNA molecule according to the invention or, in particular, DNA molecules encoding two or more mutant ion channel subunits. A variety of expression vector/host systems may be utilized to contain and express sequences encoding polypeptides of the invention. These include, but are not limited to, microorganisms such as bacteria transformed with plasmid or cosmid DNA expression vectors; yeast transformed with yeast expression vectors; insect cell systems infected with viral expression vectors (e.g., baculovirus); or mouse or other animal or human tissue cell systems. Mammalian cells can also be used to express a protein using a vaccinia virus expression system. The invention is not limited by the host cell or vector employed.

The polynucleotide sequences, or variants thereof, of the present invention can be stably expressed in cell lines to allow long term production of recombinant proteins in mammalian systems. Sequences encoding the polypeptides of the present invention can be transformed into cell lines using expression vectors which may contain viral origins of replication and/or endogenous expression elements and a selectable marker gene on the same or on a separate vector. The selectable marker confers resistance to a selective agent, and its presence allows growth and recovery of cells which successfully express the introduced sequences. Resistant clones of stably transformed cells may be propagated using tissue culture techniques appropriate to the cell type.

The protein produced by a transformed cell may be secreted or retained intracellularly depending on the sequence and/or the vector used. As will be understood by those of skill in the art, expression vectors containing polynucleotides which encode a protein may be designed to contain signal sequences which direct secretion of the protein through a prokaryotic or eukaryotic cell membrane.

In addition, a host cell strain may be chosen for its ability to modulate expression of the inserted sequences or to process the expressed protein in the desired fashion. Such modifications of the polypeptide include, but are not limited to, acetylation, glycosylation, phosphorylation, and acylation. Post-translational cleavage of a "prepro" form of the protein may also be used to specify protein targeting, folding, and/or activity. Different host cells having specific cellular machinery and characteristic mechanisms for post-translational activities (e.g., CHO or HeLa cells), are available from the American Type Culture Collection (ATCC) and may be chosen to ensure the correct modification and processing of the foreign protein.

When large quantities of the protein product of the gene are needed, such as for antibody production, vectors which direct high levels of expression of this protein may be used, such as those containing the T5 or T7 inducible bacteriophage promoter. The present invention also includes the use of the expression systems described above in generating and isolating fusion proteins which contain important functional domains of the protein. These fusion proteins are used for binding, structural and functional studies as well as for the generation of appropriate antibodies.

In order to express and purify the protein as a fusion protein, the appropriate cDNA sequence is inserted into a vector which contains a nucleotide sequence encoding another peptide (for example, glutathionine succinyl transferase). The fusion protein is expressed and recovered from prokaryotic or eukaryotic cells. The fusion protein can then be purified by affinity chromatography based upon the fusion vector sequence. The desired protein is then obtained by enzymatic cleavage of the fusion protein.

Fragments of the polypeptides of the present invention may also be produced by direct peptide synthesis

using solid-phase techniques. Automated synthesis may be achieved by using the ABI 431A Peptide Synthesizer (Perkin-Elmer). Various fragments of this protein may be synthesized separately and then combined to produce the full-length molecule.

The present invention is also concerned with polypeptides having a biological function as an ion channel in a mammal, wherein a mutation event selected from the group consisting of substitutions, deletions, truncations, insertions and rearrangements has occurred so as to affect the functioning of the ion channel. In some instances this single mutation alone will produce an epilepsy phenotype or other neuro/physiological disorders associated with ion channel dysfunction.

In the case where a single mutation alone does not produce, say, an epilepsy phenotype, there would be provided one or more additional isolated mammalian polypeptides having biological functions as part of an ion channel in a mammal, wherein a mutation event selected from the group consisting of substitutions, deletions, truncations, insertions and rearrangements has occurred so as to affect the functioning of the ion channel. The cumulative effect of the mutations in each isolated mammalian polypeptide *in vivo* being to produce epilepsy or another neuro/physiological disorder in said mammal. The mutations may be in polypeptide subunits belonging to the same ion channel as described above, but may also be in polypeptide subunits that belong to different ion channels.

Typically the mutation is an amino acid substitution and the ion channel is a voltage-gated channel such as a sodium, potassium, calcium or chloride channel or a ligand-gated channel such as a member of the nAChR/GABA super family of receptors, or a functional fragment or homologue thereof.

Mutation combinations may be selected from, but are not restricted to, those represented in Table 1.

Accordingly, in a further aspect of the present invention there is provided an isolated polypeptide, said polypeptide being a mutant or variant ion channel subunit wherein a mutation event selected from the group consisting of the mutation events set forth in the following Table:

Subunit Gene	Amino Acid Change
SCN1A	R222X
SCN1A	W384X
SCN1A	A395P
SCN1A	F403L
SCN1A	Y413N
SCN1A	V422E
SCN1A	R1407X
SCN1A	M1780T
SCN1A	R1892X
SCN1B	R85H
SCN2A	R223Q
SCN2A	V892I
SCN2A	L1003I
SCN2A	T1200A
SCN2A	R1319Q
CHRNA5	V134I
CHRNA2	A125T
CHRNA3	R37H
KCNQ2	K69fsX119
KCNQ2	M1V
KCNQ2	M1T
KCNQ2	R353G
KCNQ2	R430X
KCNQ2	R570S
KCNQ2	L619R

has occurred.

In a further aspect of the invention there is provided an isolated polypeptide, said polypeptide being a mutant or variant ion channel subunit wherein a mutation event has occurred such that the polypeptide has the amino acid sequence set forth in one of SEQ ID Numbers: 73-95. The mutation event disrupts the functioning of an ion channel so as to produce a phenotype of epilepsy, and/or one or more other disorders associated with ion channel dysfunction, including but not restricted to, hyper- or hypo-kalemic periodic paralysis, myotonias, malignant hyperthermia, myasthenia, cardiac arrhythmias, episodic

ataxia, migraine, Alzheimer's disease, Parkinson's disease, schizophrenia, hyperekplexia, anxiety, depression, phobic obsessive symptoms, neuropathic pain, inflammatory pain, chronic/acute pain, Bartter's syndrome, polycystic kidney disease, Dent's disease, hyperinsulinemic hypoglycemia of infancy, cystic fibrosis, congenital stationary night blindness and total colour-blindness.

In a particularly preferred embodiment of the present invention, the isolated polypeptide has an amino acid sequence as shown in any one of SEQ ID Numbers: 73-95. The sequences correspond to the novel amino acid changes laid out in Table 1 for those instances where the DNA mutation results in an amino acid change.

According to still another aspect of the present invention there is provided an isolated polypeptide, said polypeptide being a mutant KCNQ2 subunit, wherein the mutation event has occurred in the C-terminal domain of the KCNQ2 subunit and leads to a disturbance in the calmodulin binding affinity of the subunit, so as to produce an epilepsy phenotype.

In one form of the invention the mutations are substitutions in which an arginine residue is replaced with a glycine residue, or a leucine residue is replaced with an arginine. Preferably the substitutions are R353G and L619R transitions as illustrated by SEQ ID NOS: 92 and 95 respectively.

In a further form of the invention the mutations result in the replacement of an arginine for a stop codon, or an arginine is replaced with a serine. Preferably the mutations are R430X and R570S transitions as illustrated by SEQ ID NOS: 93 and 94 respectively.

In a still further aspect of the present invention there is provided a combination of two or more isolated polypeptides each having a novel mutation event as laid out in Table 1. The cumulative effect of the mutations in each isolated polypeptide molecule *in vivo* is to produce

an epilepsy or another disorder associated with ion channel dysfunction as described above in said mammal.

In a particularly preferred embodiment of the present invention, the isolated polypeptides have an amino acid sequence as shown in any one of SEQ ID Numbers: 73-95. The sequences correspond to the novel amino acid changes laid out in Table 1.

According to still another aspect of the present invention there is provided an isolated polypeptide comprising the amino acid sequence set forth in any one of SEQ ID Numbers: 73-95.

According to still another aspect of the present invention there is provided a polypeptide consisting of the amino acid sequence set forth in any one of SEQ ID Numbers: 73-95.

According to still another aspect of the present invention there is provided a method of preparing a polypeptide, comprising the steps of:

(1) culturing host cells transfected with an expression vector comprising a nucleic acid molecule as described above under conditions effective for polypeptide production; and

(2) harvesting the mutant ion channel subunit.

The mutant ion channel subunit may be allowed to assemble with other subunits constituting the channel that are either wild-type or themselves mutant subunits, whereby the assembled ion channel is harvested.

According to still another aspect of the invention there is provided a polypeptide which is the product of the process described above.

Substantially purified protein or fragments thereof can then be used in further biochemical analyses to establish secondary and tertiary structure. Such methodology is known in the art and includes, but is not restricted to, X-ray crystallography of crystals of the proteins or of the assembled ion channel incorporating the proteins or by nuclear magnetic resonance (NMR).

Determination of structure allows for the rational design of pharmaceuticals to interact with the ion channel as a whole or through interaction with a specific subunit protein (see drug screening below), alter the overall ion channel protein charge configuration or charge interaction with other proteins, or to alter its function in the cell.

It will be appreciated that the mutant ion channel subunits included as part of the present invention will be useful in further applications which include a variety of hybridisation and immunological assays to screen for and detect the presence of either a normal or mutated gene or gene product. The invention enables therapeutic methods for the treatment of epilepsy as well as other disorders associated with ion channel dysfunction and also enables methods for the diagnosis or prognosis of epilepsy as well as other disorders associated with ion channel dysfunction.

Therapeutic Applications

According to still another aspect of the invention there is provided a method of treating epilepsy as well as other disorders associated with ion channel dysfunction, including but not restricted to, hyper- or hypo-kalemic periodic paralysis, myotonias, malignant hyperthermia, myasthenia, cardiac arrhythmias, episodic ataxia, migraine, Alzheimer's disease, Parkinson's disease, schizophrenia, hyperekplexia, anxiety, depression, phobic obsessive symptoms, neuropathic pain, inflammatory pain, chronic/acute pain, Bartter's syndrome, polycystic kidney disease, Dent's disease, hyperinsulinemic hypoglycemia of infancy, cystic fibrosis, congenital stationary night blindness or total colour-blindness, comprising administering a selective antagonist, agonist or modulator of an ion channel or ion channel subunit, when the ion channel contains a mutation in a subunit comprising the channel, as described above, to a subject in need of such treatment. Said mutation event may be causative of the

disorder when expressed alone or when expressed in combination with one or more additional mutations in subunits of the same or different ion channels, which are typically those identified in Table 1.

In still another aspect of the invention there is provided the use of a selective antagonist, agonist or modulator of an ion channel or ion channel subunit when the ion channel contains a mutation in a subunit comprising the channel, as described above, said mutation being causative of epilepsy as well as other disorders associated with ion channel dysfunction, including but not restricted to, hyper- or hypo-kalemic periodic paralysis, myotonias, malignant hyperthermia, myasthenia, cardiac arrhythmias, episodic ataxia, migraine, Alzheimer's disease, Parkinson's disease, schizophrenia, hyperekplexia, anxiety, depression, phobic obsessive symptoms, neuropathic pain, inflammatory pain, chronic/acute pain, Bartter's syndrome, polycystic kidney disease, Dent's disease, hyperinsulinemic hypoglycemia of infancy, cystic fibrosis, congenital stationary night blindness or total colour-blindness, when expressed alone or when expressed in combination with a second mutation in a subunit of the same or different ion channel, as described above, in the manufacture of a medicament for the treatment of the disorder.

In one aspect, a suitable antagonist, agonist or modulator will restore wild-type function to the ion channel or channels containing the mutations of the present invention, or will negate the effects the mutant channel or channels have on cell function.

Using methods well known in the art, a mutant ion channel may be used to produce antibodies specific for the mutant channel that is causative of the disease or to screen libraries of pharmaceutical agents to identify those that bind the mutant ion channel.

In one aspect, an antibody, which specifically binds to a mutant ion channel or mutant ion channel subunit of

the invention, may be used directly as an agonist, antagonist or modulator, or indirectly as a targeting or delivery mechanism for bringing a pharmaceutical agent to cells or tissues that express the mutant ion channel.

In a still further aspect of the invention there is provided an antibody which is immunologically reactive with a polypeptide as described above, but not with a wild-type ion channel or ion channel subunit thereof.

In particular, there is provided an antibody to an assembled ion channel containing a mutation in a subunit comprising the channel, which is causative of epilepsy or another disorder associated with ion channel dysfunction when expressed alone or when expressed in combination with one or more other mutations in subunits of the same or different ion channels. Such antibodies may include, but are not limited to, polyclonal, monoclonal, chimeric, and single chain antibodies as would be understood by the person skilled in the art.

For the production of antibodies, various hosts including rabbits, rats, goats, mice, humans, and others may be immunized by injection with a polypeptide as described above or with any fragment or oligopeptide thereof which has immunogenic properties. Various adjuvants may be used to increase immunological response and include, but are not limited to, Freund's, mineral gels such as aluminium hydroxide, and surface-active substances such as lysolecithin. Adjuvants used in humans include BCG (bacilli Calmette-Guerin) and Corynebacterium parvum.

It is preferred that the oligopeptides, peptides, or fragments used to induce antibodies to the mutant ion channel have an amino acid sequence consisting of at least 5 amino acids, and, more preferably, of at least 10 amino acids. It is also preferable that these oligopeptides, peptides, or fragments are identical to a portion of the amino acid sequence of the natural protein and contain the entire amino acid sequence of a small, naturally occurring

molecule. Short stretches of ion channel amino acids may be fused with those of another protein, such as KLH, and antibodies to the chimeric molecule may be produced.

Monoclonal antibodies to a mutant ion channel may be prepared using any technique which provides for the production of antibody molecules by continuous cell lines in culture. These include, but are not limited to, the hybridoma technique, the human B-cell hybridoma technique, and the EBV-hybridoma technique. (For example, see Kohler et al., 1975; Kozbor et al., 1985; Cote et al., 1983; Cole et al., 1984).

Monoclonal antibodies produced may include, but are not limited to, mouse-derived antibodies, humanised antibodies and fully human antibodies.

Antibodies may also be produced by inducing *in vivo* production in the lymphocyte population or by screening immunoglobulin libraries or panels of highly specific binding reagents as disclosed in the literature. (For example, see Orlandi et al., 1989; Winter and Milstein, 1991).

Antibody fragments which contain specific binding sites for a mutant ion channel may also be generated. For example, such fragments include, F(ab')2 fragments produced by pepsin digestion of the antibody molecule and Fab fragments generated by reducing the disulfide bridges of the F(ab')2 fragments. Alternatively, Fab expression libraries may be constructed to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity. (For example, see Huse et al., 1989).

Various immunoassays may be used for screening to identify antibodies having the desired specificity. Numerous protocols for competitive binding or immunoradiometric assays using either polyclonal or monoclonal antibodies with established specificities are well known in the art. Such immunoassays typically involve the measurement of complex formation between an ion channel and its specific antibody. A two-site, monoclonal-

based immunoassay utilizing antibodies reactive to two non-interfering ion channel epitopes is preferred, but a competitive binding assay may also be employed.

In a further aspect of the invention there is provided a method of treating epilepsy as well as other disorders associated with ion channel dysfunction, including but not restricted to, hyper- or hypo-kalemic periodic paralysis, myotonias, malignant hyperthermia, myasthenia, cardiac arrhythmias, episodic ataxia, migraine, Alzheimer's disease, Parkinson's disease, schizophrenia, hyperekplexia, anxiety, depression, phobic obsessive symptoms, neuropathic pain, inflammatory pain, chronic/acute pain, Bartter's syndrome, polycystic kidney disease, Dent's disease, hyperinsulinemic hypoglycemia of infancy, cystic fibrosis, congenital stationary night blindness or total colour-blindness, comprising administering an isolated nucleic acid molecule which is the complement (antisense) of any one of the nucleic acid molecules described above and which encodes an RNA molecule that hybridizes with the mRNA encoding a mutant ion channel subunit of the invention, to a subject in need of such treatment.

In a still further aspect of the invention there is provided the use of an isolated nucleic acid molecule which is the complement (antisense) of a nucleic acid molecule of the invention and which encodes an RNA molecule that hybridizes with the mRNA encoding a mutant ion channel subunit of the invention, in the manufacture of a medicament for the treatment of epilepsy as well as other disorders associated with ion channel dysfunction, including but not restricted to, hyper- or hypo-kalemic periodic paralysis, myotonias, malignant hyperthermia, myasthenia, cardiac arrhythmias, episodic ataxia, migraine, Alzheimer's disease, Parkinson's disease, schizophrenia, hyperekplexia, anxiety, depression, phobic obsessive symptoms, neuropathic pain, inflammatory pain, chronic/acute pain, Bartter's syndrome, polycystic kidney

disease, Dent's disease, hyperinsulinemic hypoglycemia of infancy, cystic fibrosis, congenital stationary night blindness or total colour-blindness.

Typically, a vector expressing the complement (antisense) of the polynucleotides of the invention may be administered to a subject in need of such treatment. Many methods for introducing vectors into cells or tissues are available and equally suitable for use *in vivo*, *in vitro*, and *ex vivo*. For *ex vivo* therapy, vectors may be introduced into stem cells taken from the patient and clonally propagated for autologous transplant back into that same patient. Delivery by transfection, by liposome injections, or by polycationic amino polymers may be achieved using methods which are well known in the art. (For example, see Goldman *et al.*, 1997).

Additional antisense or gene-targeted silencing strategies may include, but are not limited to, the use of antisense oligonucleotides, injection of antisense RNA, transfection of antisense RNA expression vectors, and the use of RNA interference (RNAi) or short interfering RNAs (siRNA). Still further, catalytic nucleic acid molecules such as DNAzymes and ribozymes may be used for gene silencing (Breaker and Joyce, 1994; Haseloff and Gerlach, 1988). These molecules function by cleaving their target mRNA molecule rather than merely binding to it as in traditional antisense approaches.

In a further aspect, a suitable agonist, antagonist or modulator may include peptides, phosphopeptides or small organic or inorganic compounds that can restore wild-type activity of ion channels containing mutations in the subunits which comprise the channels as described above.

Peptides, phosphopeptides or small organic or inorganic compounds suitable for therapeutic applications may be identified using nucleic acids and peptides of the invention in drug screening applications as described below. Molecules identified from these screens may also be

of therapeutic application in affected individuals carrying other ion channel subunit gene mutations if the molecule is able to correct the common underlying functional deficit imposed by these mutations and those of the invention.

There is therefore provided a method of treating epilepsy as well as other disorders associated with ion channel dysfunction comprising administering a compound that is a suitable agonist, antagonist or modulator of an ion channel and that has been identified using the mutant ion channel subunits of the invention.

In some instances, an appropriate approach for treatment may be combination therapy. This may involve the administering an antibody or complement (antisense) to a mutant ion channel or ion channel subunit of the invention to inhibit its functional effect, combined with administration of wild-type ion channel subunits which may restore levels of wild-type ion channel formation to normal levels. Wild-type ion channel subunits of the invention can be administered using gene therapy approaches as described above for complement administration.

There is therefore provided a method of treating epilepsy as well as other disorders associated with ion channel dysfunction comprising administration of an antibody or complement to a mutant ion channel or ion channel subunit of the invention in combination with administration of wild-type ion channel subunits.

In still another aspect of the invention there is provided the use of an antibody or complement to a mutant ion channel or ion channel subunit of the invention in combination with the use of wild-type ion channel subunits, in the manufacture of a medicament for the treatment of epilepsy as well as other disorders associated with ion channel dysfunction.

In further embodiments, any of the agonists, antagonists, modulators, antibodies, complementary

sequences or vectors of the invention may be administered in combination with other appropriate therapeutic agents. Selection of the appropriate agents may be made by those skilled in the art, according to conventional pharmaceutical principles. The combination of therapeutic agents may act synergistically to effect the treatment or prevention of the various disorders described above. Using this approach, therapeutic efficacy with lower dosages of each agent may be possible, thus reducing the potential for adverse side effects.

Any of the therapeutic methods described above may be applied to any subject in need of such therapy, including, for example, mammals such as dogs, cats, cows, horses, rabbits, monkeys, and most preferably, humans.

Drug Screening

According to still another aspect of the invention, nucleic acid molecules of the invention as well as peptides of the invention, particularly purified mutant ion channel subunit polypeptide and cells expressing these, are useful for the screening of candidate pharmaceutical agents for the treatment of epilepsy as well as other as other disorders associated with ion channel dysfunction, including but not restricted to, hyper- or hypo-kalemic periodic paralysis, myotonias, malignant hyperthermia, myasthenia, cardiac arrhythmias, episodic ataxia, migraine, Alzheimer's disease, Parkinson's disease, schizophrenia, hyperekplexia, anxiety, depression, phobic obsessive symptoms, neuropathic pain, inflammatory pain, chronic/acute pain, Bartter's syndrome, polycystic kidney disease, Dent's disease, hyperinsulinemic hypoglycemia of infancy, cystic fibrosis, congenital stationary night blindness or total colour-blindness.

Still further, it provides the use of a polypeptide complex for the screening of candidate pharmaceutical compounds.

Still further, it provides the use wherein high throughput screening techniques are employed.

Compounds that can be screened in accordance with the invention include, but are not limited to peptides (such as soluble peptides), phosphopeptides and small organic or inorganic molecules (such as natural product or synthetic chemical libraries and peptidomimetics).

In one embodiment, a screening assay may include a cell-based assay utilising eukaryotic or prokaryotic host cells that are stably transformed with recombinant molecules expressing the polypeptides or fragments of the invention, in competitive binding assays. Binding assays will measure the formation of complexes between a specific mutant ion channel subunit polypeptide or ion channel incorporating a mutant ion channel subunit polypeptide, and the compound being tested, or will measure the degree to which a compound being tested will inhibit or restore the formation of a complex between a specific mutant ion channel subunit polypeptide or ion channel incorporating a mutant ion channel subunit polypeptide, and its interactor or ligand.

The invention is particularly useful for screening compounds by using the polypeptides of the invention in transformed cells, transfected or injected oocytes, or animal models bearing mutated ion channel subunits such as transgenic animals or gene targeted (knock-in) animals (see transformed hosts). Drug candidates can be added to cultured cells that express a single mutant ion channel subunit or combination of mutant ion channel subunits (appropriate wild-type ion channel subunits should also be expressed for receptor assembly), can be added to oocytes transfected or injected with either a mutant ion channel subunit or combination of mutant ion channel subunits (appropriate wild-type ion channel subunits must also be injected for receptor assembly), or can be administered to an animal model containing a mutant ion channel or combination of mutant ion channels. Determining the

ability of the test compound to modulate mutant ion channel activity can be accomplished by a number of techniques known in the art. These include for example measuring the effect on the current of the channel (e.g. calcium-, chloride-, sodium-, potassium-ion flux) as compared to the current of a cell or animal containing wild-type ion channels. Current in cells can be measured by a number of approaches including the patch-clamp technique (methods described in Hamill *et al*, 1981) or using fluorescence based assays as are known in the art (see Gonzalez et al. 1999). Drug candidates that alter the current to a more normal level are useful for treating or preventing epilepsy as well as other disorders associated with ion channel dysfunction.

Non cell-based assays may also be used for identifying compounds that can inhibit or restore binding between the polypeptides of the invention or ion channels incorporating the polypeptides of the invention, and their interactors. Such assays are known in the art and include for example AlphaScreen technology (PerkinElmer Life Sciences, MA, USA). This application relies on the use of beads such that each interaction partner is bound to a separate bead via an antibody. Interaction of each partner will bring the beads into proximity, such that laser excitation initiates a number of chemical reactions ultimately leading to fluorophores emitting a light signal. Candidate compounds that inhibit the binding of the mutant ion channel subunit, or ion channel incorporating the mutant subunit, with its interactor will result in loss of light emission, while candidate compounds that restore the binding of the mutant ion channel subunit, or ion channel incorporating the mutant subunit, with its interactor will result in positive light emission. These assays ultimately enable identification and isolation of the candidate compounds.

High-throughput drug screening techniques may also employ methods as described in WO84/03564. Small peptide

test compounds synthesised on a solid substrate can be assayed for mutant ion channel subunit polypeptide or mutant ion channel binding. Bound mutant ion channel or mutant ion channel subunit polypeptide is then detected by methods well known in the art. In a variation of this technique, purified polypeptides of the invention can be coated directly onto plates to identify interacting test compounds.

The invention also contemplates the use of competition drug screening assays in which neutralizing antibodies capable of specifically binding the mutant ion channel compete with a test compound for binding thereto. In this manner, the antibodies can be used to detect the presence of any peptide that shares one or more antigenic determinants of the mutant ion channel.

The polypeptides of the present invention may also be used for screening compounds developed as a result of combinatorial library technology. This provides a way to test a large number of different substances for their ability to modulate activity of a polypeptide. A substance identified as a modulator of polypeptide function may be peptide or non-peptide in nature. Non-peptide "small molecules" are often preferred for many *in vivo* pharmaceutical applications. In addition, a mimic or mimetic of the substance may be designed for pharmaceutical use. The design of mimetics based on a known pharmaceutically active compound ("lead" compound) is a common approach to the development of novel pharmaceuticals. This is often desirable where the original active compound is difficult or expensive to synthesise or where it provides an unsuitable method of administration. In the design of a mimetic, particular parts of the original active compound that are important in determining the target property are identified. These parts or residues constituting the active region of the compound are known as its pharmacophore. Once found, the pharmacophore structure is modelled according to its

physical properties using data from a range of sources including x-ray diffraction data and NMR. A template molecule is then selected onto which chemical groups which mimic the pharmacophore can be added. The selection can be made such that the mimetic is easy to synthesise, is likely to be pharmacologically acceptable, does not degrade *in vivo* and retains the biological activity of the lead compound. Further optimisation or modification can be carried out to select one or more final mimetics useful for *in vivo* or clinical testing.

It is also possible to isolate a target-specific antibody and then solve its crystal structure. In principle, this approach yields a pharmacophore upon which subsequent drug design can be based as described above. It may be possible to avoid protein crystallography altogether by generating anti-idiotypic antibodies (anti-ids) to a functional, pharmacologically active antibody. As a mirror image of a mirror image, the binding site of the anti-ids would be expected to be an analogue of the original receptor. The anti-id could then be used to isolate peptides from chemically or biologically produced peptide banks.

Another alternative method for drug screening relies on structure-based rational drug design. Determination of the three dimensional structure of the polypeptides of the invention, or the three dimensional structure of the ion channels which incorporate these polypeptides allows for structure-based drug design to identify biologically active lead compounds.

Three dimensional structural models can be generated by a number of applications, some of which include experimental models such as x-ray crystallography and NMR and/or from *in silico* studies of structural databases such as the Protein Databank (PDB). In addition, three dimensional structural models can be determined using a number of known protein structure prediction techniques based on the primary sequences of the polypeptides (e.g.

SYBYL - Tripos Associated, St. Louis, MO), *de novo* protein structure design programs (e.g. MODELER - MSI Inc., San Diego, CA, or MOE - Chemical Computing Group, Montreal, Canada) or *ab initio* methods (e.g. see US Patent Numbers 5331573 and 5579250).

Once the three dimensional structure of a polypeptide or polypeptide complex has been determined, structure-based drug discovery techniques can be employed to design biologically-active compounds based on these three dimensional structures. Such techniques are known in the art and include examples such as DOCK (University of California, San Francisco) or AUTODOCK (Scripps Research Institute, La Jolla, California). A computational docking protocol will identify the active site or sites that are deemed important for protein activity based on a predicted protein model. Molecular databases, such as the Available Chemicals Directory (ACD) are then screened for molecules that complement the protein model.

Using methods such as these, potential clinical drug candidates can be identified and computationally ranked in order to reduce the time and expense associated with typical 'wet lab' drug screening methodologies.

Compounds identified through screening procedures as described above, and which are based on the use of the mutant nucleic acid and polypeptides of the invention, can also be tested for their effect on correcting the functional deficit imposed by other gene mutations in affected individuals including other ion channel subunit mutations.

Such compounds form a part of the present invention, as do pharmaceutical compositions containing these and a pharmaceutically acceptable carrier.

Pharmaceutical Preparations

Compounds identified from screening assays and shown to restore ion channel wild-type activity can be administered to a patient at a therapeutically effective

dose to treat or ameliorate epilepsy as well as other disorders associated with ion channel dysfunction, as described above. A therapeutically effective dose refers to that amount of the compound sufficient to result in amelioration of symptoms of the disorder.

Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals. The data obtained from these studies can then be used in the formulation of a range of dosages for use in humans.

Pharmaceutical compositions for use in accordance with the present invention can be formulated in a conventional manner using one or more physiological acceptable carriers, excipients or stabilisers which are well known. Acceptable carriers, excipients or stabilizers are non-toxic at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids; antioxidants including absorbic acid; low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; binding agents including hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, arginine or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; salt-forming counterions such as sodium; and/or non-ionic surfactants such as Tween, Pluronics or polyethylene glycol (PEG).

The formulation of pharmaceutical compositions for use in accordance with the present invention will be based on the proposed route of administration. Routes of administration may include, but are not limited to, inhalation, insufflation (either through the mouth or nose), oral, buccal, rectal or parental administration.

Diagnostic and Prognostic Applications

Polynucleotide sequences encoding an ion channel subunit may be used for the diagnosis or prognosis of epilepsy, as well as other as other disorders associated with ion channel dysfunction, including but not restricted to, hyper- or hypo-kalemic periodic paralysis, myotonias, malignant hyperthermia, myasthenia, cardiac arrhythmias, episodic ataxia, migraine, Alzheimer's disease, Parkinson's disease, schizophrenia, hyperekplexia, anxiety, depression, phobic obsessive symptoms, neuropathic pain, inflammatory pain, chronic/acute pain, Bartter's syndrome, polycystic kidney disease, Dent's disease, hyperinsulinemic hypoglycemia of infancy, cystic fibrosis, congenital stationary night blindness or total colour-blindness, and the use of the nucleic acid molecules incorporated as part of the invention in diagnosis or prognosis of these disorders, or a predisposition to these disorders, is therefore contemplated. The nucleic acid molecules incorporating the novel mutation events laid out in Table 1 may be used for this purpose.

The polynucleotides that may be used for diagnostic or prognostic purposes include oligonucleotide sequences, genomic DNA and complementary RNA and DNA molecules. The polynucleotides may be used to detect and quantitate gene expression in biological samples. Genomic DNA used for the diagnosis or prognosis may be obtained from body cells, such as those present in the blood, tissue biopsy, surgical specimen, or autopsy material. The DNA may be isolated and used directly for detection of a specific sequence or may be amplified by the polymerase chain reaction (PCR) prior to analysis. Similarly, RNA or cDNA may also be used, with or without PCR amplification. To detect a specific nucleic acid sequence, hybridisation using specific oligonucleotides, restriction enzyme digest and mapping, PCR mapping, RNAse protection, and various other methods may be employed. Oligonucleotides specific

to particular sequences can be chemically synthesized and labelled radioactively or nonradioactively and hybridised to individual samples immobilized on membranes or other solid-supports or in solution. The presence, absence or excess expression of any one of the mutant ion channel genes of the invention may then be visualized using methods such as autoradiography, fluorometry, or colorimetry.

In a further diagnostic or prognostic approach, the nucleotide sequences of the invention may be useful in assays that detect the presence of associated disorders, particularly those mentioned previously. The nucleotide sequences may be labelled by standard methods and added to a fluid or tissue sample from a patient under conditions suitable for the formation of hybridisation complexes. After a suitable incubation period, the sample is washed and the signal is quantitated and compared with a standard value. If the amount of signal in the patient sample is significantly altered in comparison to a control sample then the presence of altered levels of nucleotide sequences in the sample indicates the presence of the associated disorder. Such assays may also be used to evaluate the efficacy of a particular therapeutic treatment regimen in animal studies, in clinical trials, or to monitor the treatment of an individual patient.

In order to provide a basis for the diagnosis or prognosis of epilepsy and other disorders as described above, which are associated with the ion channel subunit mutations or variants of the invention, the nucleotide sequence of each gene can be compared between normal tissue and diseased tissue in order to establish whether the patient expresses a mutant gene.

In order to provide a basis for the diagnosis or prognosis of a disorder associated with abnormal expression of an ion channel subunit gene of the invention, a normal or standard profile for expression is established. This may be accomplished by combining body

fluids or cell extracts taken from normal subjects, either animal or human, with a sequence, or a fragment thereof, encoding the relevant ion channel subunit gene, under conditions suitable for hybridisation or amplification. Standard hybridisation may be quantified by comparing the values obtained from normal subjects with values from an experiment in which a known amount of a substantially purified polynucleotide is used. Another method to identify a normal or standard profile for expression of an ion channel subunit gene is through quantitative RT-PCR studies. RNA isolated from body cells of a normal individual is reverse transcribed and real-time PCR using oligonucleotides specific for the relevant gene is conducted to establish a normal level of expression of the gene. Standard values obtained in both these examples may be compared with values obtained from samples from patients who are symptomatic for a disorder. Deviation from standard values is used to establish the presence of a disorder.

Once the presence of a disorder is established and a treatment protocol is initiated, hybridisation assays or quantitative RT-PCR studies may be repeated on a regular basis to determine if the level of expression in the patient begins to approximate that which is observed in the normal subject. The results obtained from successive assays may be used to show the efficacy of treatment over a period ranging from several days to months.

According to a further aspect of the invention there is provided the use of a polypeptide as described above in the diagnosis or prognosis of epilepsy as well as other disorders associated with ion channel dysfunction, including but not restricted to, hyper- or hypo-kalemic periodic paralysis, myotonias, malignant hyperthermia, myasthenia, cardiac arrhythmias, episodic ataxia, migraine, Alzheimer's disease, Parkinson's disease, schizophrenia, hyperekplexia, anxiety, depression, phobic obsessive symptoms, neuropathic pain, inflammatory pain,

chronic/acute pain, Bartter's syndrome, polycystic kidney disease, Dent's disease, hyperinsulinemic hypoglycemia of infancy, cystic fibrosis, congenital stationary night blindness or total colour-blindness.

When a diagnostic or prognostic assay is to be based upon proteins constituting an ion channel, a variety of approaches are possible. For example, diagnosis or prognosis can be achieved by monitoring differences in the electrophoretic mobility of normal and mutant proteins that form the ion channel. Such an approach will be particularly useful in identifying mutants in which charge substitutions are present, or in which insertions, deletions or substitutions have resulted in a significant change in the electrophoretic migration of the resultant protein. Alternatively, diagnosis or prognosis may be based upon differences in the proteolytic cleavage patterns of normal and mutant proteins, differences in molar ratios of the various amino acid residues, or by functional assays demonstrating altered function of the gene products.

In another aspect, antibodies that specifically bind mutant ion channels may be used for the diagnosis or prognosis of a disorder, or in assays to monitor patients being treated with a complete ion channel or agonists, antagonists, modulators or inhibitors of an ion channel. Antibodies useful for diagnostic or prognostic purposes may be prepared in the same manner as described above for therapeutics. Diagnostic or prognostic assays for ion channels include methods that utilize the antibody and a label to detect a mutant ion channel in human body fluids or in extracts of cells or tissues. The antibodies may be used with or without modification, and may be labelled by covalent or non-covalent attachment of a reporter molecule.

A variety of protocols for measuring the presence of mutant ion channels, including but not restricted to, ELISAs, RIAs, and FACS, are known in the art and provide a

basis for diagnosing or prognosing a disorder. The expression of a mutant ion channel or combination of mutant ion channels is established by combining body fluids or cell extracts taken from test mammalian subjects, preferably human, with antibody to the ion channel or channels under conditions suitable for complex formation. The amount of complex formation may be quantitated by various methods, preferably by photometric means. Antibodies specific for the mutant ion channels will only bind to individuals expressing the said mutant ion channels and not to individuals expressing only wild-type channels (ie normal individuals). This establishes the basis for diagnosing the disorder.

Once an individual has been diagnosed or prognosed with a disorder, effective treatments can be initiated as described above. Treatments can be directed to amend the combination of ion channel subunit mutations or may be directed to one mutation.

Microarray

In further embodiments, complete cDNAs, oligonucleotides or longer fragments derived from any of the polynucleotide sequences described herein may be used as probes in a microarray. The microarray can be used to diagnose or prognose epilepsy, as well as other disorders associated with ion channel dysfunction, through the identification of genetic variants, mutations, and polymorphisms in the ion channel subunits that form part of the invention, to understand the genetic basis of a disorder, or can be used to develop and monitor the activities of therapeutic agents.

According to a further aspect of the present invention, tissue material obtained from genetically modified non-human animal models generated as a result of the identification of specific ion channel subunit human mutations (see below), particularly those disclosed in the present invention, can be used in microarray experiments.

These experiments can be conducted to identify the level of expression of specific ion channel subunits, or the level of expression of any cDNA clone from whole-tissue libraries, in diseased tissue as opposed to normal control tissue. Variations in the expression level of genes, including ion channel subunits, between the two tissues indicates their possible involvement in the disease process either as a cause or consequence of the original ion channel subunit mutation present in the animal model. These experiments may be used to determine gene function, to understand the genetic basis of a disorder, to diagnose or prognose a disorder, and to develop and monitor the activities of therapeutic agents. Microarrays may be prepared, used, and analyzed using methods known in the art. (For example, see Schena et al., 1996; Heller et al., 1997).

Transformed Hosts

The present invention also provides for the production of genetically modified (knock-out, knock-in and transgenic), non-human animal models comprising nucleic acid molecules containing the novel ion channel mutations or variants as laid out in Table 1. These animals are useful for the study of the function of ion channels, to study the mechanisms by which combinations of mutations in ion channel subunits interact to give rise to disease and the effects of these mutations on tissue development, for the screening of candidate pharmaceutical compounds, for the creation of explanted mammalian cell cultures which express mutant ion channels or combinations of mutant ion channels, and for the evaluation of potential therapeutic interventions.

Animal species which are suitable for use in the animal models of the present invention include, but are not limited to, rats, mice, hamsters, guinea pigs, rabbits, dogs, cats, goats, sheep, pigs, and non-human primates such as monkeys and chimpanzees. For initial

studies, genetically modified mice and rats are highly desirable due to the relative ease in generating knock-in, knock-out or transgenics of these animals, their ease of maintenance and their shorter life spans. For certain studies, transgenic yeast or invertebrates may be suitable and preferred because they allow for rapid screening and provide for much easier handling. For longer term studies, non-human primates may be desired due to their similarity with humans.

To create an animal model for a mutated ion channel, or an animal model incorporating a combination of mutations, several methods can be employed. These include, but are not limited to, generation of a specific mutation in a homologous animal gene, insertion of a wild type human gene and/or a humanized animal gene by homologous recombination, insertion of a mutant (single or multiple) human gene as genomic or minigene cDNA constructs using wild type or mutant or artificial promoter elements, or insertion of artificially modified fragments of the endogenous gene by homologous recombination. The modifications include insertion of mutant stop codons, the deletion of DNA sequences, or the inclusion of recombination elements (lox p sites) recognized by enzymes such as Cre recombinase.

To create transgenic mice in order to study gain of gene function *in vivo*, any mutant ion channel subunit gene of the invention can be inserted into a mouse germ line using standard techniques such as oocyte microinjection. Gain of gene function can mean the over-expression of a gene and its protein product, or the genetic complementation of a mutation of the gene under investigation. For oocyte injection, one or more copies of the mutant gene can be inserted into the pronucleus of a just-fertilized mouse oocyte. This oocyte is then reimplanted into a pseudo-pregnant foster mother. The live-born mice can then be screened for integrants using analysis of tail DNA for the presence of the relevant

human ion channel subunit gene sequence. The transgene can be either a complete genomic sequence injected as a YAC, BAC, PAC or other chromosome DNA fragment, a cDNA with either the natural promoter or a heterologous promoter, or a minigene containing all of the coding region and other elements found to be necessary for optimum expression.

To generate knock-out mice or knock-in mice, gene targeting through homologous recombination in mouse embryonic stem (ES) cells may be applied. Knock-out mice are generated to study loss of gene function *in vivo* while knock-in mice (which are preferred) allow the study of gain of function or to study the effect of specific gene mutations. Knock-in mice are similar to transgenic mice however the integration site and copy number are defined in the former.

For knock-out mouse generation, gene targeting vectors can be designed such that they delete (knock-out) the protein coding sequence of the relevant ion channel subunit gene in the mouse genome. In contrast, knock-in mice can be produced whereby a gene targeting vector containing the relevant ion channel subunit gene can integrate into a defined genetic locus in the mouse genome. For both applications, homologous recombination is catalysed by specific DNA repair enzymes that recognise homologous DNA sequences and exchange them via double crossover.

Gene targeting vectors are usually introduced into ES cells using electroporation. ES cell integrants are then isolated via an antibiotic resistance gene present on the targeting vector and are subsequently genotyped to identify those ES cell clones in which the gene under investigation has integrated into the locus of interest. The appropriate ES cells are then transmitted through the germline to produce a novel mouse strain.

In instances where gene ablation results in early embryonic lethality, conditional gene targeting may be employed. This allows genes to be deleted in a temporally

and spatially controlled fashion. As above, appropriate ES cells are transmitted through the germline to produce a novel mouse strain, however the actual deletion of the gene is performed in the adult mouse in a tissue specific or time controlled manner. Conditional gene targeting is most commonly achieved by use of the cre/lox system. The enzyme cre is able to recognise the 34 base pair loxP sequence such that loxP flanked (or floxed) DNA is recognised and excised by cre. Tissue specific cre expression in transgenic mice enables the generation of tissue specific knock-out mice by mating gene targeted floxed mice with cre transgenic mice. Knock-out can be conducted in every tissue (Schwenk et al., 1995) using the 'deleter' mouse or using transgenic mice with an inducible cre gene (such as those with tetracycline inducible cre genes), or knock-out can be tissue specific for example through the use of the CD19-cre mouse (Rickert et al., 1997).

Once knock-in animals have been produced which contain a specific mutation in a particular ion channel subunit, mating combinations may be initiated between such animals so as to produce progeny containing combinations of two or more ion channel mutations. These animals effectively mimic combinations of mutations that are proposed to cause human IGE cases. These animal models can subsequently be used to study the extent and mechanisms of disease as related to the mutated ion channel combinations, as well as for the screening of candidate therapeutic compounds.

According to still another aspect of the invention there is provided the use of genetically modified non-human animals as described above for the screening of candidate pharmaceutical compounds (see drug screening above). These animals are also useful for the evaluation (eg therapeutic efficacy, toxicity, metabolism) of candidate pharmaceutical compounds, including those identified from the invention as described above, for the

treatment of epilepsy as well as other as other disorders associated with ion channel dysfunction as described above.

It will be clearly understood that, although a number of prior art publications are referred to herein, this reference does not constitute an admission that any of these documents forms part of the common general knowledge in the art, in Australia or in any other country.

Throughout this specification and the claims, the words "comprise", "comprises" and "comprising" are used in a non-exclusive sense, except where the context requires otherwise.

It will be apparent to the person skilled in the art that while the invention has been described in some detail for the purposes of clarity and understanding, various modifications and alterations to the embodiments and methods described herein may be made without departing from the scope of the inventive concept disclosed in this specification.

Brief Description of the Drawings

Preferred forms of the invention will now be described, by way of example only, with reference to the following examples and the accompanying drawings, in which:

Figure 1 provides an example of ion channel subunit stoichiometry and the effect of multiple versus single ion channel subunit mutations. Figure 1A: A typical channel may have five subunits of three different types. Figure 1B: In outbred populations complex diseases such as idiopathic generalized epilepsies may be due to mutations in two (or more) different subunit genes. Because only one allele of each subunit gene is abnormal, half the expressed subunits will have the mutation. Figure 1C: In inbred populations, both alleles of a single subunit gene will be affected, so all expressed subunits will be mutated. Figure 1D: Autosomal dominant disorders can be

attributed to single ion channel subunit mutations that give rise to severe functional consequences.

Figure 2 represents the location of mutations identified in the KCNQ2 ion channel subunit constituting the potassium channel. M: Missense mutation; T: Truncation mutation; F: Frameshift mutation; S: Splice site mutation.

Figure 3 provides examples of epilepsy pedigrees where mutation profiles of ion channel subunits for individuals constituting the pedigree have begun to be determined. These examples have been used to illustrate how the identification of novel ion channel subunit mutations and variations in IGE individuals can combine to give rise to the disorder.

Figure 4 shows the results of yeast two-hybrid analysis of R353G and L619R KCNQ2 mutants. Yeast were transformed with the empty DB (BAIT) plasmid (DBLeu), DB-Q2C wt, DB-Q2C R353G mutant or the DB-Q2 L619R mutant as indicated in A and the AD-CaM (TARGET) vector was introduced by gap-repair. Yeast control strains (Invitrogen™) were included on all plates for comparison. Control 1 has no interaction. Control 2 has a weak interaction. Control 3 has a moderately strong interaction. Control 4 has a strong interaction and control 5 has a very strong interaction. B. Growth of transformed yeast and controls on -leu -tryp selection. Yeast can grow on -leu if they contain the DB plasmid, and -tryp if they have AD plasmid. C. Growth of transformed yeast and controls on -leu -tryp -his +40mM 3AT after 48hrs. Yeast can grow on -his+3AT if the *his* reporter gene is activated by interaction between the BAIT and TARGET plasmids. D-F. *LacZ* Filter assay for interaction between BAIT and TARGET plasmids, photos taken after 2hrs (D), 7hrs (E) and 24hrs (F). Activation of the β-*galactosidase* reporter gene by interaction of the BAIT and TARGET plasmids leads to the dark appearance of colonies.

Figure 5 shows the results of CaM affinity experiments with the R353G and L619R KCNQ2 mutants. The

chart below shows the values from the CPRG assay for β-galactosidase activity as a measure of KCNQ2C-CaM binding efficiency. The area of each bar in the chart equates to the CaM binding efficiency of the BAIT. Broken lines indicate statistical comparison by Student's *t* test * $P<0.01$, ** $P<0.001$.

Modes for Performing the Invention

Potassium channels are the most diverse class of ion channel. The *C. elegans* genome encodes about 80 different potassium channel genes and there are probably more in mammals. About ten potassium channel genes are known to be mutated in human disease and include four members of the KCNQ gene sub-family of potassium channels. KCNQ proteins have six transmembrane domains, a single P-loop that forms the selectivity filter of the pore, a positively charged fourth transmembrane domain that probably acts as a voltage sensor, and intracellular amino and carboxy termini. The C-terminus is long and contains a conserved "A domain" followed by a short stretch thought to be involved in subunit assembly.

Four KCNQ subunits are thought to combine to form a functional potassium channel. All five known KCNQ proteins can form homomeric channels *in vitro* and the formation of heteromers appears to be restricted to certain combinations. For instance KCNQ2 and KCNQ3, which are predominantly expressed in the central nervous system, form a heteromultimeric channel that mediates the neuronal muscarinic-regulated current (M-current), also known as the M-channel (or M-type K^+ channel). The M-current is a slowly activating, non-inactivating potassium conductance known to regulate neuronal excitability by determining the firing properties of neurons and their responsiveness to synaptic input (Wang et al., 1998). Because it is the only current active at voltages near the threshold for action potential initiation, the M-current has a major impact on neuronal excitability.

Sodium (the alpha subunit) and calcium channels are thought to have evolved from the potassium channel subunit, and they each consist of four domains covalently linked as the one molecule, each domain being equivalent to one of the subunits that associate to form the potassium channel. Each of the four domains of the sodium and calcium channels are comprised of six transmembrane segments.

Voltage-gated sodium channels are required to generate the electrical excitation in neurones, heart and skeletal muscle fibres, which express tissue specific isoforms. Sodium channels are heteromers of a pore forming alpha subunit and a modulatory beta-1 subunit, with an additional beta-2 subunit in neuronal channels. Ten genes encoding sodium channel alpha subunits and 3 genes encoding different beta subunits have so far been identified. The beta subunits of the sodium channels do not associate with the alpha subunits to form any part of the pore, they do however affect the way the alpha pore forming subunit functions.

As with sodium channels, calcium channels consist of a single pore forming alpha subunit, of which at least six types have been identified to date, and several accessory subunits including four beta, one gamma and one alpha2-delta gene. Many of these subunits also encode multiple splice variants adding to the diversity of receptor subunits of this family of ion channels.

The ion channels in the nAChR/GABA super family show a theoretical pentameric channel. Gamma-Aminobutyric acid (GABA) is the most abundant inhibitory neurotransmitter in the central nervous system. GABA-ergic inhibition is mediated by two major classes of receptors, type A (GABA-A) and type B (GABA-B). GABA-B receptors are members of the class of receptors coupled to G-proteins and mediate a variety of inhibitory effects via secondary messenger cascades. GABA-A receptors are ligand-gated chloride channels that mediate rapid inhibition.

The GABA-A channel has 16 separate, but related, genes encoding subunits. These are grouped on the basis of sequence identity into alpha, beta, gamma, delta, epsilon, theta and pi subunits. There are six alpha subunits (α1-α6), three beta subunits (β1-β3) and three gamma subunits (γ1-γ3). Each GABA-A receptor comprises five subunits which may, at least in theory, be selected from any of these subunits.

Neuronal nicotinic acetylcholine receptors (nAChRs) consist of heterologous pentamers comprising various combinations of alpha subunits or alpha and beta subunits (α2-α9; β2-β4). The alpha subunits are characterised by adjacent cysteine residues at amino acid positions 192 and 193, and the beta subunits by the lack of these cysteine residues. They are ligand-gated ion channels differentially expressed throughout the brain to form physiologically and pharmacologically distinct receptors hypothesised to mediate fast, excitatory transmission between neurons of the central nervous system or to modulate neurotransmission from their presynaptic position.

In chicken and rat, the predominant nAChR subtype is composed of alpha-4 and beta-2 subunits. The transmembrane 2 (M2) segments of the subunits are arranged as alpha helices and contribute to the walls of the neurotransmitter-gated ion channel. The alpha helices appear to be kinked and orientated in such a way that the side chains of the highly conserved M2-leucine residues project inwards when the channel is closed. ACh is thought to cause a conformational change by altering the association of the amino acid residues of M2. The opening of the channel seems to be due to rotations of the gate forming side chains of the amino acid residues; the conserved polar serines and threonines may form the critical gate in the open channel.

Example 1: Identification of mutations in ion channels

Previous studies by reference (Wallace et al., 1998; PCT/AU01/00581; Wallace et al., 2001b; Australian patent AU-B-56247/96; Steinlein et al., 1995; PCT/AU01/00541; Phillips et al., 2001; PCT/AU01/00729; PCT/AU01/01648; PCT/AU02/00910; Wallace et al., 2001a, the disclosures of which are incorporated herein by reference) have identified mutations in a number of ion channel subunits associated with epilepsy. These include ion channel subunits of voltage-gated (eg SCN1A, SCN1B, KCNQ2, KCNQ3) or ligand-gated (eg CHRNA4, CHRNB2, GABRG2, GABRD) types. To identify further mutations in ion channel genes, subunits which comprise the ion channels were screened for molecular defects in epilepsy patients.

Human genomic sequence available from the Human Genome Project was used to characterize the genomic organisation for each subunit gene. Each gene was subsequently screened for sequence changes using single strand conformation polymorphism (SSCP) analysis in a large sample of epileptics with common sporadic IGE subtypes eg juvenile myoclonic epilepsy (JME), childhood absence epilepsy (CAE), juvenile absence epilepsy (JAE) and epilepsy with generalized tonic-clonic seizures (TCS). Clinical observations can then be compared to the molecular defects characterized in order to establish the combinations of mutant subunits involved in the various disease states, and therefore to provide validated drug targets for each of these disease states. This will provide a basis for novel drug treatments directed at the genetic defects present in each patient.

The coding sequence for each of the ion channel subunits was aligned with human genomic sequence present in available databases at the National Centre for Biotechnology Information (NCBI). The BLASTN algorithm was typically used for sequence alignment and resulted in the genomic organisation (intron-exon structure) of each gene being determined. Where genomic sequence for an ion

channel subunit was not available, BACs or PACs containing the relevant ion channel subunit were identified through screening of high density filters containing these clones and were subsequently sequenced.

Availability of entire genomic sequence for each ion channel subunit facilitated the design of intronic primers spanning each exon. These primers were used for both high throughput SSCP screening and direct DNA sequencing.

Example 2: Sample preparation for SSCP screening

A large collection of individuals affected with epilepsy have undergone careful clinical phenotyping and additional data regarding their family history has been collated. Informed consent was obtained from each individual for blood collection and its use in subsequent experimental procedures. Clinical phenotypes incorporated classical IGE cases as well as GEFS+ and febrile seizure cases.

DNA was extracted from collected blood using the QIAamp DNA Blood Maxi kit (Qiagen) according to manufacturers specifications or through procedures adapted from Wyman and White (1980). Stock DNA samples were kept at a concentration of 1 ug/ul.

In preparation for SSCP analysis, samples to be screened were formatted into 96-well plates at a concentration of 30 ng/ul. These master plates were subsequently used to prepare exon specific PCR reactions in the 96-well format.

Example 3: Identification of sequence alterations in ion channel genes

SSCP analysis of specific ion channel exons followed by sequencing of SSCP bandshifts was performed on individuals constituting the 96-well plates to identify sequence alterations.

Primers used for SSCP were labelled at their 5′ end with HEX and typical PCR reactions were performed in a

total volume of 10 µl. All PCR reactions contained 67 mM Tris-HCl (pH 8.8); 16.5 mM $(NH_4)_2SO_4$; 6.5 µM EDTA; 1.5 mM $MgCl_2$; 200 µM each dNTP; 10% DMSO; 0.17 mg/ml BSA; 10 mM β-mercaptoethanol; 5 µg/ml each primer and 100 U/ml *Taq* DNA polymerase. PCR reactions were typically performed using 10 cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds followed by 25 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds. A final extension reaction for 10 minutes at 72°C followed.

Ten to twenty µl of loading dye comprising 50% (v/v) formamide, 12.5 mM EDTA and 0.02% (w/v) bromophenol blue were added to completed reactions which were subsequently run on non-denaturing 4% polyacrylamide gels with a cross-linking ratio of 35:1 (acrylamide:bis-acrylamide) and containing 2% glycerol. Gel thickness was 100µm, width 168mm and length 160mm. Gels were run at 1200 volts and approximately 20mA, at 18°C and analysed on the GelScan 2000 system (Corbett Research, Australia) according to manufacturers specifications.

PCR products showing a conformational change were subsequently sequenced. This first involved re-amplification of the amplicon from the relevant individual (primers used in this instance did not contain 5′ HEX labels) followed by purification of the PCR amplified templates for sequencing using QiaQuick PCR preps (Qiagen) based on manufacturers procedures. The primers used to sequence the purified amplicons were identical to those used for the initial amplification step. For each sequencing reaction, 25 ng of primer and 100 ng of purified PCR template were used. The BigDye sequencing kit (ABI) was used for all sequencing reactions according to the manufacturers specifications. The products were run on an ABI 377 Sequencer and analysed using the EditView program.

Table 1 shows the novel sequence changes identified in the ion channel subunits screened.

Example 4: Digenic model examples

In some instances a single mutation in an ion channel alone is insufficient to give rise to an epilepsy phenotype. However combinations of mutations each conferring a subtle change of function to an ion channel, as proposed by the digenic model (PCT/AU01/00872), may be sufficient to produce an epilepsy phenotype.

Using mutations and variations in ion channel subunits previously identified, the digenic model may be validated through a parametric analysis of large families in which two abnormal alleles co-segregate by chance to identify mutations which act co-operatively to give an epilepsy phenotype. It is envisaged that the strategy of careful clinical phenotyping in these large families, together with a linkage analysis based on the digenic hypothesis will allow identification of the mutations in ion channels associated with IGEs. If molecular genetic studies in IGE are successful using the digenic hypothesis, such an approach might serve as a model for other disorders with complex inheritance.

The digenic hypothesis predicts that the closer the genetic relationship between affected individuals, the more similar the sub-syndromes, consistent with published data (Italian League Against Epilepsy Genetic Collaborative Group, 1993). This is because more distant relatives are less likely to share the same combinations of mutated subunits.

Identical twins have the same pair of mutated subunits and the same minor alleles so the sub-syndromes are identical. Affected sib-pairs, including dizygous twins, with the same sub-syndrome would also have the same pair of mutated subunits, but differences in minor alleles would lead to less similarity than with monozygous twins. Some sib-pairs and dizygous twins, have quite different sub-syndromes; this would be due to different combinations of mutated subunits, when the parents have more than two mutated alleles between them.

A special situation exists in inbred communities that parallels observations on autosomal recessive mouse models. Here the two mutated alleles of the digenic model are the same and thus result in a true autosomal recessive disorder. Because all affected individuals have the same pair of mutated alleles, and a similar genetic background, the phenotypes are very similar.

In outbred communities approximately 1% of the population would have IGE genotypes (2 mutated alleles) and 0.3% would clinically express IGE. Most of these would have mutations in two different channel subunits. In such communities most cases would appear "sporadic" as the risk to first degree relatives would be less than 10%.

For example, let there be three IGE loci (A,B,C) and let the frequency of abnormal alleles (a*,b*,c*) at each locus be .027 and of normal alleles (a, b, c) be .973. Then, the distribution of genotypes aa*, a*a, a*a* and aa at locus A will be .0263 (.027 x .973), .0263, .0007 and .9467 respectively, and similarly for loci B and C. In this population .8485 will have no mutated alleles ($.9467^3$), .1413 will have one mutated allele (a* or b* or c*; .0263 x $.9467^2$ x 6), .0098 will have two abnormal alleles (.0020 two same abnormal alleles, .0078, two different abnormal alleles) and 0.00037 will have more than two abnormal alleles. Thus in this population .01, or 1%, will have two or more abnormal alleles (IGE genotype), and the total abnormal allele frequency will be .08 (3 x .027).

To determine the familial risks and allele patterns in affected pairs, the frequency distribution of population matings and the percentage of children with 2 or more abnormal alleles must be determined. The frequency of matings with no abnormal alleles (0 x 0) is .72 ($.8485^2$), for 1 x 0 and 0 x 1 matings .24 (2 x .8485 x .1413), for a 1 x 1 mating .020, and for 2 x 0 and 0 x 2 matings .0166 etc. From this distribution of matings the frequency of children with 2 or more abnormal alleles can

be shown to be .01. For example, the 0 x 2 and 2 x 0 matings contribute .0033 of this .01 frequency (.0166 [mating frequency] x .2 [chance of that mating producing a child with 2 or more abnormal alleles]).

To determine parental risk it can be shown that of children with 2 abnormal alleles (IGE genotype), .49 derive from 1 x 1 matings where no parent is affected, .33 derive from a 2 x 0 and 0 x 2 matings etc. For the 2 x 0 and 0 x 2 matings, half the parents have IGE genotypes and contribute .16 (.33/2) to the parental risk with the total parental risk of an IGE genotype being .258. The other matings that contribute to affected parent-child pairs are 2 x 1, 1 x 2, 3 x 0, 0 x 3 etc.

The sibling risk of an IGE genotype is .305. For example 2 x 0 and 0 x 2 matings contributed .08 to the sibling risk (.33[fraction of children with 2 abnormal alleles] x .25[the chance of that mating producing a child with 2 or more abnormal alleles]). Similarly the offspring risk was determined to be .248 by mating individuals with 2 abnormal alleles with the general population. Thus at 30% penetrance the risk for IGE phenotype for parents of a proband is .077, for siblings .091, and for offspring .074.

It can be shown that affected sib pairs share the same abnormal allele pair in 85% of cases. This is because of all affected sib pairs 44% derive from 1 x 1 matings and 23% from 0 x 2 and 2 x 0 matings where all affected siblings have the same genotype. In contrast, 24% derive from 1 x 2 matings and 9% from 3 x 1 and 2 x 2 matings etc where affected sibling genotypes sometimes differ.

For affected parent-child pairs, genotypes are identical in only 58%. Of affected parent child pairs, 43% derive from 0 x 2 matings where gentoypes are identical, whereas 38% derive from 0 x 3 and 17% from 1 x 2 where the majority of crosses yield different affected genotypes.

Based on the digenic model it has been postulated that most classical IGE and GEFS$^+$ cases are due to the

combination of two mutations in multi-subunit ion channels. These are typically point mutations resulting in a subtle change of function. The critical postulate is that two mutations, usually, but not exclusively, in different subunit alleles ("digenic model"), are required for clinical expression of IGE.

The hypothesis that similar phenotypes can be caused by the combination of mutations in two (or more) different subunits (outbred communities), or by the same mutation in two (or more) alleles of the same subunit (inbred communities), may seem implausible. However, applying the digenic hypothesis to the theoretical pentameric channel shown in Figure 1, in outbred communities IGE will be due to subunit combinations such as $\alpha^*\alpha\beta^*\beta\Delta$, $\alpha^*\alpha\beta\beta\Delta^*$ or $\alpha\alpha\beta^*\beta\Delta^*$ (mutated subunits indicated by *). In inbred communities $\alpha^*\alpha^*\beta\beta\Delta$ or $\alpha\alpha\beta^*\beta^*\Delta$ combinations might cause IGE phenotypes. We assume that the mutations will not cause reduced expression of the alleles and that the altered ion channel excitability, and consequent IGE phenotype, caused by mutations in two different alleles is similar to that caused by the same mutation in both alleles of one subunit. Finally, subunit mutations with more severe functional consequences (eg breaking a disulphide bridge in SCN1B or amino acid substitution in the pore forming regions of SCN1A for GEFS$^+$) cause autosomal dominant generalized epilepsies with a penetrance of 60-90%. Such "severe" mutations are rare (allele frequency <0.01%) and are infrequent causes of GEFS$^+$. They very rarely, or perhaps never, cause classical IGE.

The relative separate segregation of classical IGE and GEFS$^+$ phenotypes is an anecdotal clinical observation of ours (Singh et al., 1999), although the separation is not absolute. The separation is supported by previous family and EEG studies of Doose and colleagues who described "type A" and "type B" liabilities which we may approximate the GEFS$^+$ and classical IGE groupings respectively (Doose and Baier, 1987).

The digenic model predicts that affected sib pairs will share the same genes in 85% of cases whereas they will have at least one different allele in the remaining 15%. In contrast, only 58% of parent-child pairs share the same alleles in a 3 locus model. Thus there should be greater similarity of syndromes between sibling pairs than parent-child pairs. This would be most objectively measured by age of onset and seizure types.

Estimates for the risk of febrile seizures or IGE in relatives vary. The estimates range from 5%-10% for siblings, 4%-6% for offspring, 3%-6% for parents, and 2-3% for grandparents. Underestimation may occur because IGE manifest in youth, and parents and particularly grandparents may be unaware of seizures in themselves in younger years. This is particularly true where there was stigma associated with epilepsy and where the epilepsy may have been mild and unrecognized. Underestimation of sibling and offspring risks occurs when unaffected young children are counted, some of whom will develop IGE in adolescence. Overestimation may occur with misdiagnosis of seizures or inclusion of seizures unrelated to IGE (e.g. due to trauma or tumors)

In autosomal dominant models the risk to affected relatives reduces proportionally (50% for first degree relatives, 25% for second degree etc). For all oligogenic or polygenic models the risk decreases more quickly. For a digenic model with three loci, the risks are 9.1% for siblings, 7.4% for offspring, 7.7% for parents. Rigorous measurement of the familial recurrence rates, with careful phenotyping and age-corrected risk estimates could be compared with the predictions from the digenic model, and it is proposed to do this.

There is a small amount of information on IGE families regarding haplotype distribution. For example, there is some evidence for a locus on 8q as determined by parametric linkage in a single family (Fong et al., 1998) and by non-parametric analysis in multiple small families

(Zara et al., 1995). Interestingly, in the latter study the 8q haplotype not infrequently came from the unaffected parent. This would be quite compatible with the digenic model and evaluation of other data sets in this manner could be used to test the hypothesis, and it is proposed to do this.

Following the analysis of one large family with epilepsy where the two main phenotypes were childhood absence epilepsy (CAE) and febrile seizures (FS), the inheritance of FS was found to be autosomal dominant and the penetrance 75%. However the inheritance of CAE in this family was not simple Mendelian, but suggestive of complex inheritance with the involvement of more than one gene. The power of this large family was used to explore the complex genetics of CAE further.

Linkage analysis on this family in which individuals with CAE, FS and FS+ were deemed affected led to the detection of linkage on chromosome 5q and identification of a mutation in the GABRG2 gene (R43Q) which is localised to this region (Wallace et al., 2001a; PCT/AU01/00729). All 10 tested individuals with FS alone in this family had this mutation and 7 CAE affected individuals in this family also had the mutation. To test the digenic model of IGEs in the CAE affected individuals, the whole genome screen of this family was reanalysed with only individuals with CAE considered affected. Linkage analysis was performed using FASTLINK v4.0, two-point lod scores were calculated assuming 50% penetrance and a 2% phenocopy rate and individuals with FS or FS+ were coded as unknown. Markers producing a lod score greater than 1 were reanalysed without a phenocopy rate and at the observed penetrance for CAE in this family (30%). Results from the analysis revealed significant linkage to chromosome 14q22-q23 (lod 3.4). This provides strong evidence for a second locus segregating with CAE affected individuals in this family. While the GABRG2 mutation is sufficient to cause FS, the CAE phenotype is thought to be due to both the

GABRG2 mutation and a mutation occurring in a gene mapping to the 14q locus, as proposed by the digenic model.

For the application of the digenic model to sporadic cases of IGE and affected individuals belonging to smaller families in which genotyping and linkage analysis is not a feasible approach to disease gene identification, direct mutation analysis of ion channel genes in these individuals has been carried out as described above. In Table 1 there is provided an indication of novel genetic alterations so far identified through mutation analysis screening of these individuals. Figure 2 provides an example to indicate where some of these mutations have occurred with respect to the potassium channel KCNQ2 gene.

The identification of novel mutations and variations in ion channel subunits in IGE individuals provides resources to further test the digenic hypothesis and mutation profiles are starting to accumulate for a number of subunit changes that are observed in the same individuals. Figure 3 provides results from some of these profiles.

Figure 3A shows a 3 generation family in which individual III-1 has myoclonic astatic epilepsy and contains a N43del mutation in the SCN3A gene as well as an A1067T mutation in the SCN1A gene. Individual I-1 also has the SCN3A mutation but alone this mutation is not sufficient to cause epilepsy in this individual. The SCN3A mutation has likely been inherited from the grandfather through the mother, while the SCN1A mutation is likely to arise from the father. Both parents are unaffected but have yet to be screened for the presence of the mutations in these subunits. Individual II-1 is likely to contain an as yet unidentified ion channel subunit mutation acting in co-operation with the SCN3A mutation already identified in this individual.

Figure 3B is another 3 generation family in which individual III-1 has myoclonic astatic epilepsy due to a combination of the same SCN3A and SCN1A mutations as

above. However, in this family both parents have febrile seizures most likely due to the presence of just one of the mutations in each parent, as proposed by the model. This is in contrast to individuals II-2 and II-3 in Figure 4A who also contain one of the mutations in these genes each. These individuals are phenotypically normal most likely due to incomplete penetrance of these mutations in each case.

Figure 3C shows a larger multi-generation family in which individual IV-5 has a mutation in both the SCN3A and GABRG2 subunits. In combination, these give rise to severe myoclonic epilepsy of infancy but alone either cause febrile seizures (GABRG2 mutation in III-3 and IV-4) or are without an effect (SCN3A mutation in III-2) as proposed by the model.

These examples therefore illustrate the digenic model as determined from mutation analysis studies of ion channel subunits in affected individuals and highlight the need to identify genetic alterations in the genes encoding ion channel subunits.

Example 5: Analysis of ion channels and ion channel subunits

The structure and function of the mutant ion channels and mutant ion channel subunits of the present invention can be determined using a variety of molecular biological studies. These studies may provide clues as to the mechanisms by which mutations in ion channel subunits effect the functioning of the ion channel. For instance the identification of proteins that interact with mutant ion channels (or whose interaction is impeded by a mutation in an ion channel subunit) may help determine the molecular mechanisms that are disrupted as a result of a mutation. Procedures such as the yeast two-hybrid system can be used to discover and identify such interacting proteins.

The principle behind the yeast two-hybrid procedure is that many eukaryotic transcriptional activators, including those in yeast, consist of two discrete modular domains. The first is a DNA-binding domain that binds to a specific promoter sequence and the second is an activation domain that directs the RNA polymerase II complex to transcribe the gene downstream of the DNA binding site. Both domains are required for transcriptional activation as neither domain can activate transcription on its own. In the yeast two-hybrid procedure, the gene of interest or parts thereof (BAIT), is cloned in such a way that it is expressed as a fusion to a peptide that has a DNA binding domain. A second gene, or number of genes, such as those from a cDNA library (TARGET), is cloned so that it is expressed as a fusion to an activation domain. Interaction of the protein of interest with its binding partner brings the DNA-binding peptide together with the activation domain and initiates transcription of the reporter genes. The first reporter gene will select for yeast cells that contain interacting proteins (this reporter is usually a nutritional gene required for growth on selective media). The second reporter is used for confirmation and while being expressed in response to interacting proteins it is usually not required for growth.

KCNQ2 interactors

Despite the identification of a number of KCNQ2 mutations responsible for epilepsy, including those of the present study, the underlying biological mechanisms responsible for the epilepsy remains largely uncharacterized. Towards identifying these mechanisms, the large intracellular C-terminal region of KCNQ2 was screened for interactions with other proteins using the yeast-two hybrid procedure. The C-terminus accounts for 63% of the KCNQ2 protein and, in common with other KCNQ subunits, contains a conserved 'A domain' (Jentsch, 2000; Schwake et al., 2000) thought to be involved in subunit

interactions as well as another distal short conserved region that has been associated with subunit assembly, at least in KCNQ1 (Jentsch, 2000; Schmitt et al., 2000).

A) Yeast-two hybrid analysis

A yeast two-hybrid screen was carried out using the ProQuest™ Two-Hybrid System with Gateway™ Technology (Invitrogen™) according to manufacturer's directions. A KCNQ2 C-terminal entry (BAIT) clone was generated using the pENTR Directional TOPO® Cloning Kit (Invitrogen™). The following primers were designed to amplify the intracellular C-terminal region of KCNQ2 based on the sequence of human KCNQ2 (Genbank accession number NM_172107): KCNQ2F: 5'-CACCAAGGTTCAGGAGCAGCACAGG-3' and KCNQ2R: 5'-TCACTTCCTGGGCCCGGCCCAGCC-3'. The 1611 base pair cloned fragment included exon 10a (found in all our amplified clones), corresponding to amino acid 373-382 of the KCNQ2 protein. The extra 30 base pairs (10 amino acids) were included in our numbering. The PCR-product was cloned into the pENTR/D-TOPO® vector (Invitrogen™) via the TOPO® Cloning reaction according to the manufacturer's instructions. Following sequence verification, the KCNQ2 cDNA fragment was then subcloned into pDEST™32, the DNA Binding domain (DB) Gateway™ Destination Vector (Invitrogen™).

The ProQuest™ Two-Hybrid human brain cDNA Library (TARGET) with Gateway™ technology (ResGen™, Invitrogen™ Corporation) was amplified according to the manufacturer's instructions. Plasmid DNA was purified from the cell pellet using the HiSpeed Plasmid Maxi Kit (Qiagen) according to the manufacturer's instructions.

Both the DBLeu (empty bait vector) and DB-KCNQ2 wild-type (wt) C-term BAITS were transformed into the yeast strain Mav203 and plated onto minimal selective media lacking leucine. A duplicate was carried out where the empty library TARGET (pAD) vector was co-transformed in addition to each BAIT and plated onto minimal selective

media lacking leucine (-leu) and tryptophan (-tryp). Yeast control strains (Invitrogen™) were included on all plates. Control 1, used as a negative control, contained empty plasmids pPC97 and pPC86. Control 2 had pPC97-RB and pPC86-E2F1, which express a relatively weak interaction. Control 3 contained plasmids encoding the *Drosophila* DP (pPC97) and E2F (pPC86) domains that have a moderately strong interaction, and provide a control for plasmid shuffling. Control 4 contained pPC97-Fos and pPC86-Jun which express a relatively strong interaction, and control 5 had a pCL1 plasmid encoding full-length GAL4p and empty pPC86 and was used as a positive control.

The constructs were tested for self-activation of the *his* and β-*gal* reporter genes according to Invitrogen™ instructions.

For the yeast-two hybrid screen, competent yeast cells were prepared for each BAIT (DB-KCNQ2 wt C-term construct) to be screened, transformed with 31μg of ProQuest™ Two-Hybrid human brain AD (activation domain)-cDNA Library and plated onto minimal selective media lacking leucine (-leu), tryptophan (-tryp) and histidine (-his) and containing 3-aminotriazole (+3AT). Positive colonies from each screen were PCR-amplified and re-introduced into fresh yeast cells containing the BAIT to re-test for two-hybrid interaction phenotypes. Those giving rise to more than one PCR product or that failed to re-test positively were systematically eliminated. Positives that re-tested were sequenced using the ABI PRISM® BigDye™ Terminators v3.0 technology. Once identified, the sequence of the potential interactor was checked to verify it was in the same translational frame as the Gal4p-AD encoding sequence of the prey construct.

Approximately 3 x 10^6 clones from the ProQuest™ Two-Hybrid human brain cDNA Library were screened for interaction with the DB-Q2C wt bait. Among 1039 positive AD-cDNAs recovered, re-tested and subsequently sequenced

all were identified as the CALM2 gene, encoding the ubiquitous, Ca^{2+}-binding protein, Calmodulin (CaM).

The interaction between the C-terminal region of KCNQ2 and CaM has also been reported by other studies (Wen and Levitan, 2002; Yus-Najera et al., 2002; Gamper and Shapiro, 2003). In mammals, the CaM protein is coded by a multigene family consisting of three bona fide members, CALM1, CALM2 and CALM3. Within the non-coding regions of the CaM transcripts, no striking homology is observed, and codon usage is maximally divergent amongst the three CaM mRNAs that encode an identical protein. It has been hypothesised that the existence of a multigene family provides a tight and complex level of regulatory control at the level of gene expression (Palfi et al., 2002). CaM genes are differentially expressed in the CNS during development and differential regulation of the CaM genes appears necessary to maintain the temporal and spatial fidelity of the CaM protein levels in all subcellular domains. Besides the fundamental housekeeping functions associated with CaM, it is also involved in specialized neuronal functions, such as the synthesis and release of neurotransmitters, neurite extension, long-term potentiation and axonal transport (Palfi et al., 2002).

B) Effect of epilepsy-associated KCNQ2 mutations on the CaM-KCNQ2 interaction

To assess the effect that the C-terminus mutations of the present invention had on CaM binding, two of the identified mutations (R353G and L619R) were introduced into the DB-Q2C construct by mutagenesis and were re-analysed for an interaction with CaM using the yeast two-hybrid procedure.

The following primers were used to incorporate the c1057C→G (R353G) and c1856T→G (L619R) changes into the pDEST™32- KCNQ2 C-terminal bait construct.

R353G F 5′-CGCCACCAACCTCTCGGGCACAGACCTGCACTC-3′

R353G R 5'-GAGTGCAGGTCTGTGCCCGAGAGGTTGGTGGCG-3'
L619R F 5'-CTTGTCCATGGAGAAGAAGCGGGACTTCCTGGTGAATATC-3'
L619R R 5'-GATATTCACCAGGAAGTCCCGCTTCTTCTCCATGGACAAG-3'

Overlapping PCR products were generated using the TOPO® cloning compatible KCNQ2F primer from the initial cloning and the mutagenesis reverse primers, and the KCNQ2R primer from the initial cloning with the mutagenesis forward primers. Products were gel extracted and purified before a second round of PCR using the initial KCNQ2 F&R primers. These products were also gel extracted before cloning into the pDEST™32 bait vector via the TOPO® system (as described above). Mutant baits were sequence verified.

The interaction between each DB-Q2C mutant and CaM was then tested by the yeast two-hybrid assay and compared to the interaction with DB-Q2 wt. Three different PCR-amplified CaM positive clones from the initial screen were re-introduced by gap-repair[20] into the prey vector (pPC86) in the yeast strain expressing either DB-Q2C wt, DB-Q2C mutants or the empty DBLeu vector, used as negative control.

CaM interaction with the DB-Q2C wt and mutants was then assessed by expression of the *HIS3* and *LacZ* reporter genes.

The Q2C R353G mutant did not interact with CaM, as seen by no growth on *HIS3* selective plate (Figure 4C) and no blue readout in the *LacZ* filter assay (seen as dark squares in Figure 4D-F). On the other hand, the DB-Q2C L619R mutant was shown to still interact with CaM, as seen by growth on *HIS3* selective plate (Figure 4C) and the blue readout in the *LacZ* filter assay. Interestingly, the DB-Q2C L619R mutant showed an even greater growth level on *HIS3* selective plate than the DB-Q2C wt and also appeared to stain faster and more intensely blue in the *LacZ* filter assay, suggesting a stronger interaction between CaM and this mutant.

In order to better quantify β-gal activity, a second assay was carried out using the high sensitivity substrate Chlorophenol Red-β-D-Galactopyranoside (CPRG) in liquid culture. The affinity of the DB-Q2C/AD-CaM interaction was measured in terms of units of β-gal activity, with a zero value indicating no expression of the *LacZ* reporter gene, and hence no interaction.

In the CPRG assay, a value of 0.05 units β-gal activity (Figure 5) was significantly different from the empty bait vector replicate ($P<0.01$, Student's *t* test), confirming the interaction of the DB-Q2C wt with CaM.

As observed in the *LacZ* filter assay, the CPRG assay showed a significant difference in the interaction between the Q2C R353G mutant and CaM as compared to the wt replicate ($P<0.01$, Student's *t* test, Figure 4).

These results suggest that the R353G mutation alters the structural conformation of the KCNQ2 C-terminal domain such that it is no longer able to bind to CaM and that this single point mutation is sufficient to abolish the interaction. By abolishing CaM binding, the R353G mutation could lead to an impairment of M-current *in vivo* due to decreased opening of the channel.

In contrast, the CPRG assay for the L619R Q2C mutant showed a significantly higher level of β-gal activity units (0.26 units) than the wt replicate ($P<0.001$, Student's *t* test, Figure 5). This finding indicates that the L619R mutation alters the conformation of the protein in a manner that increases CaM binding affinity for the KCNQ2 C-terminal domain by approximately 5-fold. The increased affinity for CaM may affect the ability of the complex to change conformation normally in response to calcium signalling. Alternatively, the marked increase in binding of CaM to the KCNQ2 L619R mutant channel may be detrimental to the M-channel function via disruption of the normal neuronal inhibitory/excitatory balance, therefore causing the seizures associated with epilepsy, particularly BFNS. CaM is known to be involved in both the

excitatory and inhibitory neurotransmission pathways (Ohya and Botstein, 1994) and it has been proposed that the temporal and spatial restrictions on CaM itself could enable the tight control of these opposing reactions (Toutenhoofd and Strehler, 2000). Hence, the KCNQ2 L619R mutation could lead to a disruption of the local CaM pool consequently disturbing the finely balanced excitatory and inhibitory neurotransmission systems.

These results implicate CaM in the pathogenesis of epilepsy and specifically in the BFNS syndrome. Whilst further work will be required to fully elucidate the involvement of the KCNQ2-CaM interaction in neuronal excitability and its correlation with idiopathic epilepsy, these data suggest that dysfunction of this interaction leads to aberrant neuronal excitability in some BFNS patients.

The calmodulin gene (and other ion channel interacting genes) may therefore be a target for mutation in epilepsy as well as other disorders associated with ion channel dysfunction. A mutation in an ion channel interacting gene when expressed alone, or when expressed in combination with one or more other ion channel mutations or ion channel interacting gene mutations (based on the digenic model), may give rise to the disorder. The nature of the ion channel interacting genes and proteins can be studied such that these partners can also be targets for drug discovery.

Industrial Applicability

The mutant ion channel receptor subunits of the invention are useful in the diagnosis and treatment of diseases such as epilepsy and disorders associated with ion channel dysfunction including, but not limited to, hyper- or hypo-kalemic periodic paralysis, myotonias, malignant hyperthermia, myasthenia, cardiac arrhythmias, episodic ataxia, migraine, Alzheimer's disease, Parkinson's disease, schizophrenia, hyperekplexia,

anxiety, depression, phobic obsessive symptoms, neuropathic pain, inflammatory pain, chronic/acute pain, Bartter's syndrome, polycystic kidney disease, Dent's disease, hyperinsulinemic hypoglycemia of infancy, cystic fibrosis, congenital stationary night blindness and total colour-blindness.

TABLE 1

Examples of mutations and variations identified in ion channel subunit genes

Subunit Gene	Exon/Intron	DNA Mutation	Amino Acid Change	SEQ ID NOS
Sodium Channel Subunits				
Coding exonic variants – amino acid change				
SCN1A[r]	Exon 5	c664C→T	R222X	1, 73
SCN1A[r]	Exon 8	c1152G→A	W384X	2, 74
SCN1A[r]	Exon 9	c1183G→C	A395P	3, 75
SCN1A[r]	Exon 9	c1207T→C	F403L	4, 76
SCN1A[r]	Exon 9	c1237T→A	Y413N	5, 77
SCN1A[r]	Exon 9	c1265T→A	V422E	6, 78
SCN1A[r]	Exon 21	c4219C→T	R1407X	7, 79
SCN1A[r]	Exon 26	c5339T→C	M1780T	8, 80
SCN1A[r]	Exon 26	c5674C→T	R1892X	9, 81
SCN1B[r]	Exon 3	c254G→A	R85H	10, 82
SCN2A[r]	Exon 6A	c668G→A	R223Q	11, 83
SCN2A[r]	Exon 16	c2674G→A	V892I	12, 84
SCN2A[r]	Exon 17	c3007C→A	L1003I	13, 85
SCN2A[r]	Exon 19	c3598A→G	T1200A	14, 86
SCN2A[r]	Exon 20	c3956G→A	R1319Q	15, 87
Coding exonic variants – no amino acid change				
SCN2A[c]	Exon 12	c1785T→C	-	16
SCN2A[c]	Exon 27	c4919T→A	-	17
Non-coding variants				
SCN1A[r]	Intron 9	IVS9-1G→A	-	18
SCN1A[c]	Intron 23	IVS23+33G→A	-	19
SCN2A[r]	Intron 7	IVS7+61T→A	-	20
SCN2A[r]	Intron 19	IVS19-55A→G	-	21
SCN2A[r]	Intron 22	IVS22-31A→G	-	22
SCN2A[c]	Intron 2	IVS2-28G→A	-	23
SCN2A[c]	Intron 8	IVS8-3T→C	-	24
SCN2A[c]	Intron 11	IVS11+49A→G	-	25
SCN2A[c]	Intron 11	IVS11-16C→T	-	26
SCN2A[c]	Intron 17	IVS17-71C→T	-	27
SCN2A[c]	Intron 17	IVS17-74delG	-	28
SCN2A[c]	Intron 17	IVS17-74insG	-	29
Nicotinic Acetylcholine Receptor Subunits				
Coding exonic variants – amino acid change				
CHRNA5[r]	Exon 4	c400G→A	V134I	30, 88
CHRNA2[c]	Exon 4	c373G→A	A125T	31, 89
CHRNA3[c]	Exon 2	c110G→A	R37H	32, 90
Coding variants – no amino acid change				
CHRNA2[c]	Exon 4	c351C→T	-	33
CHRNA2[c]	Exon 5	c771C→T	-	34
CHRNA3[c]	Exon 2	c159A→G	-	35
CHRNA3[c]	Exon 4	c291G→A	-	36
CHRNA3[c]	Exon 4	c345G→A	-	37

TABLE 1 (Continued)

Examples of mutations and variations identified in ion channel subunit genes

Subunit Gene	Exon/Intron	DNA Mutation	Amino Acid Change	SEQ ID NOS
Non-coding variants				
CHRNA2[c]	Intron 3	IVS3-16C→T	-	38
CHRNA3[c]	Intron 3	IVS3-5T→C	-	39
CHRNA3[c]	Intron 4	IVS4+8G→C	-	40
Potassium Channel Subunits				
Coding exonic variants – amino acid change				
KCNQ2[r]	Exon 1	c204-c205insC	K69fsX119	41, 91
KCNQ2[r]	Exon 1	c1A→G	M1V	42
KCNQ2[r]	Exon 1	c2T→C	M1T	43
KCNQ2[r]	Exon 8	c1057C→G	R353G	44, 92
KCNQ2[r]	Exon 11	c1288C→T	R430X	45, 93
KCNQ2[r]	Exon 14	c1710A→T	R570S	46, 94
KCNQ2[r]	Exon 15	c1856T→G	L619R	47, 95
Non-coding variants				
KCNQ2[r]	Intron 9	IVS9+(46-48)delCCT	-	48
KCNQ3[r]	Intron 11	IVS11+43G→A	-	49
KCNQ3[c]	Intron 12	IVS12+29G→A	-	50
GABA Receptor Subunits				
Coding exonic variants – no amino acid change				
GABRB1[r]	Exon 5	c508C→T	-	51
GABRB1[r]	Exon 9	c1329G→A	-	52
GABRB1[c]	Exon 8	c975C→T	-	53
GABRG3[c]	Exon 8	c995T→C	-	54
Non-coding variants				
GABRA1[c]	5' UTR	c-142A→G	-	55
GABRA1[c]	5' UTR	c-31C→T	-	56
GABRA2[c]	3' UTR	c1615G→A	-	57
GABRA5[c]	5' UTR	c-271G→C	-	58
GABRA5[c]	5' UTR	c-228A→G	-	59
GABRA5[c]	5' UTR	c-149G→C	-	60
GABRB2[b]	5' UTR	c-159C→T	-	61
GABRB2[c]	3' UTR	c1749C→T	-	62
GABRPi[c]	5' UTR	c-101C→T	-	63
GABRB1[c]	Intron 1	IVS1+24T→G	-	64
GABRB1[c]	Intron 5	IVS6+72T→G	-	65
GABRB1[c]	Intron 7	IVS7-34A→G	-	66
GABRB3[r]	Intron 1	IVS1-14C→T	-	67
GABRB3[r]	Intron 7	IVS7+58delAA	-	68
GABRD[r]	Intron 6	IVS6+132insC	-	69
GABRD[r]	Intron 6	IVS6+130insC		70
GABRD[r]	Intron 6	IVS6+73del CGCGCCCACCGCCCCTTCCGCG	-	71
GABRG3[c]	Intron 8	IVS8-102C→T	-	72

Note: [r] Mutations or variations only occurring in individuals with epilepsy; [b] Variant seen only in normal control samples; [c] Mutations or variants seen in individuals with epilepsy as well as normal control samples. The KCNQ2 numbering is based on the large isoform (inclusion of exon 10a). The numbering of exons and introns for SCN2A is based on the publication of Kasai et al., 2001.

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